School of Biomedical Sciences

Inheritance of Fibre colour in Alpacas: Identifying the Genes Involved

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Declaration of Authenticity

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: Natasha Jeela

Date: 17.12.2014

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Abstract

Alpacas produce a high value fibre that is increasingly sought-after by the luxury fibre industry. The colour of the fibre is an important factor for alpaca breeders as it impacts the value of the fleece. Alpacas display a huge range of natural colour variants, which is part of the growing appeal of alpaca fibre, due to the increasing demand for natural fibres. White fibre is, however, still the most sought after due to its suitability for dyeing. However, the industry is currently unable to produce enough high quality fibre of any colour to meet the market demand. The knowledge surrounding fibre colour inheritance in alpacas is incomplete and as yet there is no universally accepted theory that fully explains all colours in the species. The alpaca fibre industry would benefit greatly from genetic studies that provide advanced information about breeding for fibre colour as opposed to the current system that is based only on observed phenotype, which can be inaccurate and subjective.

Three pigment genes were investigated in this project for associations with fibre colour variation, *MC1R*, *ASIP* and *Tyrp1*. These genes have been identified in other mammalian species as the major genetic determinants for black, brown and red/yellow pigment types. The aim of this project was to identify polymorphisms within these major pigment genes in alpacas that were associated with fibre colour variation and to elucidate the basis of fibre colour inheritance in this species. The method of phenotype classification employed in this project focused on the underlying pigment type causing the phenotypes rather than just on a visual observation of phenotype. It also took into account all pigmented areas of the animal, including skin colour. This approach highlighted mutations linked to pigmentation that had previously been overlooked by other groups.

A total of 37 polymorphisms were identified in *MC1R*, *ASIP* and *Tyrp1* in alpacas, with six of these polymorphisms being linked to fibre colour variation. These six are the p.Thr28Ala, p.Arg301Cys and c.222_225del in *MC1R* and the p.Arg98Cys, p.Arg118His and a p.Cys109_Arg127del in *ASIP*. No *Tyrp1* polymorphisms associated with colour variation were identified in this study.

Two of the *MC1R* polymorphisms were further investigated by testing their effect on MC1R function in an *in vitro* assay. This work provided further evidence to support the conclusions that the MC1R polymorphisms were loss of function mutations responsible for pheomelanic phenotypes in alpacas. Functional effects of *ASIP* polymorphisms were inferred by investigation of the predicted protein and known folding models structural/function relationships. These polymorphisms were predicted to result in loss of function of the ASIP protein, resulting in black fibre colour. No association was found between *Tyrp1* polymorphisms and a eumelanic brown phenotype. Chemical analysis of fibre samples was performed in conjunction with genetic analysis to further quantify the type of pigment responsible for observed phenotypes of animals included in this study. The results provided supporting evidence for the hypothesis that *Tyrp1* brown does not exist in alpacas.

The results reported in this thesis provide a better understanding of the major loci controlling pigment production in alpacas. This project successfully identified key mutations in *MC1R* and *ASIP* that lead to fibre colour variations in alpacas. These findings will have a real-world impact for breeders, and contribute to a greater understanding of colour inheritance in this species. It is expected that these results will form the basis of coat colour tests for alpacas that can be utilised by the alpaca industry to increase the number of animals of a desired colour to meet the demand for alpaca fibre, while also retaining the genetic diversity and fibre quality characteristics that may otherwise be bred out when breeding solely for colour.

Keywords: alpaca, *MC1R*, *ASIP*, *Tyrp1*, fibre pigmentation, colour, melanin

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Abbreviations

α-MSH	alpha-Melanocyte Stimulating Hormone
ASIP	Agouti Signalling Protein
BMP	bone morphogenic protein
Bp	base pairs (nucleotides)
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
cDNA	coding deoxyribonucleic acid
cM	centimorgan
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleotide triphosphate
dGTP	deoxyguanosine triphosphate
DHI	dihydroxyindol
DHICA	dihydroxyindol-carboxylic acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide tri-phosphate
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid (disodium salt)
EGF	Epidermal growth factor
g, mg, µg, ng	gram, milligram, microgram, nanogram
GPCR	G protein coupled receptor
ICK	Inhibitor cysteine knot
IPTG	isopropyl β-D-1 thiogalactopyranoside
LB	Luria Bertani
LBA	Luria Bertani broth with Agar
M, mM, µM, pM	molar, millimolar, micromolar, picomolar
MATP	Membrane associated transporter protein
MC1R	Melanocortin receptor 1
Min, hrs	minute, hours
ml, µl	millilitre, microlitre
mm, µm, nm	millimetre, micrometre, nanometre

mmol, µmol	millimole, micromole
MSA	multiple sequence alignment
NA	Not applicable
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RE	restriction enzyme
s	Seconds
SIFT	Scale-invariant feature transform
SNP	single nucleotide polymorphism
SOC	Super Optimal broth with Catabolite repression medium
SP	Signal peptide
TAE	tris acetate EDTA buffer
TE	Tris-EDTA
Tm	Melting temperature
TMD	Transmembrane domain
Tris.HCl	Tris Hydrochloride
TRP	Tyrosine related protein
Try	Tyrosine
Tryp1	Tyrosinase related protein 1
Tryp2	Tyrosinase related protein 2
UTR	untranslated region
UV	ultraviolet
V, kV, mV	volts, kilovolts, millivolts
v/v	volume/volume
w/v	weight/volume
Wnt	Wingless integration site
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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Literature Review

1.1 Alpacas, a brief history

Alpacas *(Vicugña pacos,* Kadwell 2001*)* originate from South America where they are bred for both their meat and fibre, and were highly prized by the ancient Incans (Wheeler, 1995). Alpacas with high quality fibre in pure black, brown and white were also specifically breed for ritual sacrifices (J. Wheeler, *pers. comm.*). Alpaca fibre is classed as a luxury animal fibre and is renowned for its excellent thermal properties and strength, while still being a soft and lightweight natural fibre (Australian Alpaca Association, 2006, Lupton et al., 2006, McGregor, 2006). It also has the benefit of being available in a large variety of natural colours; ranging from pure white through fawn, a range of browns to true black (Australian Alpaca Association, 2006, Lupton et al., 2006).

Domesticated over 6000 years ago, the alpaca is a member of the Camelid family; which also includes Camels *(Camelus bactrianus/dromedarius* Linnaeus, 1758*)*, Llamas *(Lama glama* Linnaeus, 1758*)*, Vicuñas *(Vicugña vicugña* Molina, 1782*)* and Guanacos *(Lama guanicoe* Mijller, 1776*)*. Vicuñas and Guanacos are wild species, while alpacas and llamas are their domesticated relatives (Kadwell et al., 2001). In the past there has been considerable debate as to the origins of the alpaca and whether they were descended from the guanaco, the vicuña or were a llama-vicuña hybrid. Following the work of Kadwell and colleagues in 2001 it is now widely accepted that alpacas are descended from the vicuña and hence have been assigned the scientific name *Vicugna pacos* (Kadwell et al., 2001).

In pre-Hispanic times the alpaca was a highly prized animal, which in addition to providing meat and fibre was used in ancient Incan rituals and formed an integral part of their culture. However the arrival of the Spanish conquistadors in the 16th century proved to be catastrophic for the species. It is estimated that within a century of the Spaniards arrival, the numbers of alpacas and llamas in Peru had dwindled to less than 10 percent of their pre-conquest numbers (Flores Ochoa, 1977, Flores Ochoa, 1982). The alpaca, an animal with enormous value to the Incans, was perceived as competition for grazing land for the more familiar sheep the Spaniards had brought with them, and their potential value was overlooked. Many of the finest animals were slaughtered for their meat while others were

relegated as pack animals in the silver and gold mines. The alpaca population declined significantly and many of the finest quality animals were lost from the gene pool. In addition, disease and war had ravaged the inhabitants of Peru, demolishing the local population and along with them much of the breeding knowledge was lost (Wheeler et al., 1995). Recent discoveries of mummified pre-conquest alpacas and llamas in Peru have revealed that these ancient animals not only possessed fleeces much finer than we see today, but also produced a much higher quantity of fibre (Wheeler et al., 1995, Wachtel, 1977).

1.1.1 The Australian alpaca industry

It is estimated that almost 90% of the world alpaca population resides in South America. However several other countries including Australia, which currently has an estimated >200,000 animals, are poised to become major contributors to the alpaca fibre market (Australian Alpaca Fleece Ltd, 2008, Adams, 2013). Alpacas were successfully introduced to Australia in the late 1980s, and are currently raised for their luxury fibre and the sale of their offspring (Frank et al., 2006, McGregor, 2006). There is also an emerging market for alpaca meat in Australia, which will be key to maintaining a sustainable industry once commercial farming of alpacas is established (Honan, 2013, Bell, 2011).

The modern alpaca industry began in Australia in the late 1980s with a small number of animals imported into the country, which formed the first breeding stock of these animals (Tuckwell, 1994, Australian Alpaca Association, 2000). Prior to this the exportation of alpacas from Peru had been banned in order to preserve the Peruvian alpaca population, which is considered as an import national resource and part of the countries heritage. However the very first importation of alpacas into Australia occurred in 1858 where a small herd was illegally smuggled out of South America and brought to NSW by Charles Ledger. However once in Australia their numbers decreased steadily where by in 1880 none of these animals were reported to have survived (Tuckwell, 1994). More recent imports of high quality alpacas from Peru have improved the genetic diversity of the Australian herd (Department of Agriculture Fisheries and Forestry, 2005). The Australia alpaca industry today is working to establish a successful fibre industry capable of producing high volume of high quality fibre to compete on the global textile market.

Alpaca fibre is in high demand in the textile industry where it is incorporated in high fashion garments, carpets and bedding. At present white/light fawn alpaca fibre attracts the highest prices, with coloured fibre commanding an average of 42% of non coloured fibre prices (Australia Alpaca Fleece Ltd, 2012). It is, however, important to maintain the natural colour variation exhibited in alpacas as there is a niche market for naturally coloured fibre (2012, McGregor, 2006). These natural fibre colours are sought-after because dyeing processes and the use of harsh chemicals, similar to those used in wool dyeing, can be avoided (McGregor, 2006, Herring, 2006)

While the price and desirability of alpaca fibre is heavily influenced by fibre colour, other characteristics including fibre type, length, diameter, evenness and yield are also important (Frank et al., 2006, Moore et al.). Alpaca fibre research has been initially focused on these fibre quality characteristics because of their commercial significance (Russel, 1994, McGregor, 2006, Frank et al., 2006, Cervante et al., 2009, Aylan-Parker and McGregor, 2002). The demand for alpaca fibre far outweighs supply with Australian breeders struggling to produce adequate volumes of fibre of consistent quality and colour (McGregor, 2006, Australian Alpaca Fleece Ltd, 2008, Calderwood, 2014, Bell, 2011). Australia has a small but rapidly growing alpaca fibre industry, focused on optimising fibre production; however commercial fibre producing herds have not yet been established in Australia.

1.1.2 The future of alpaca fibre production

Alpacas exhibit a wide variety of natural fibre colours and patterns, which is one of the factors that make the determination of the underlying colour genetics so difficult in this species. It is difficult to assign a precise colour phenotype to specific animals, mostly because of the range of intermediate shades and the inconsistency of colour interpretation (Hart, 2001, Paul, 2006). Additionally, colour descriptions used by breeders often have no relation to the underlying genetics causing the colour. The lack of knowledge surrounding fibre colour inheritance in this species has substantially hindered the expansion of the commercial white herd in Australia. White fibre is likely to always be favoured for commercial fibre herds, as it is the most profitable fibre; however, it is not

beneficial to breed out the other colours that make alpaca fibre such a unique and highly desirable natural fibre. Being able to determine fibre colour inheritance is essential information for the industry because it would enable breeders to select more effective matings in relation to colour, and move towards breeding a herd that is better suited to the market demand.

1.2 The Mammalian Pigmentary System

Fibre colour variation in mammals has long been of interest to scientists. Pigmentation in mammals has many functions including photo protection (Hearing, 2000), mate selection (Safran and McGraw, 2004, Protas and Patel, 2008) and predator avoidance (Protas and Patel, 2008, Slagsvolt, 1995). As colour mutations are easily identifiable and usually not lethal it is also an ideal system for studying gene function and interactions. Pigment studies on mice have formed much of the basis of our knowledge of the genetic basis of mammalian pigment variation (Silvers, 1979). In mice 378 genes have been identified with a role in pigmentation, almost half of these have been cloned and characterized (http://www.espcr.org/micemut/). The mouse model has served as a useful platform for analysing fibre colour variation in other species because of the significant homology of pigmentation genes among mammals. Genes that have been associated with specific phenotypes in mice have been shown to produce similar phenotypes in other species (Jackson, 1997a) and so can be useful when utilising a candidate gene approach to studying pigment in other species.

Melanogenesis is a complex and tightly regulated process that controls the production of pigment (Seiji et al., 1961, Hearing and Tsukamoto, 1991, Schallreuter, 2007, Park et al., 2010). The melanocyte is the pigment-producing cell found in the skin and hair follicle of mammals. Development of the melanocyte begins in early embryogenesis where melanocyte precursor cells, known as melanoblasts, begin the process of cell line specification in the neural crest (Thomas and Erickson, 2008). From here, the immature cells follow specific migratory patterns, where they differentiate and proliferate to eventually invade throughout the epidermis and the epidermal hair follicle (Mayer, 1973, Nishimura et al., 2002, Wilkie et al., 2002). Within the melanocyte, pigment production is confined to a highly specialised organelle known as a melanosome (Seiji et al.,

1961, Anderson, 1997, Slominski et al., 2004). Skin and hair become pigmented when the mature melanosome is transported into the surrounding keratinocytes (Lin and Fisher, 2007, Pajak et al., 1983, Scott, 2006). Mammals produce two types of pigment: the red/yellow pheomelanin and the brown/black eumelanin, and the amount and distribution of these is responsible for the variety of colour phenotypes mammals exhibit (Hearing and Tsukamoto, 1991, Prota, 1992, Ito, 1993).

1.2.1 Neural crest

Neural crest cells are a group of pluripotent cells arising in the neural tube during embryogenesis. Neural crest cells migrate and differentiate to give rise to pigment cells as well as varied cell types including those of the peripheral and enteric nervous system, adipose tissue, bone, and cartilage (Gilbert, 2000, Anderson, 1997, Shakhova and Sommer, 2010). Neural crest cells can be divided into early and late migratory types. Early migration neural crest cells differentiate into peripheral nerves, craniofacial bones, the heart and the adrenal medulla. Late migratory cells form melanoblasts, the precursor of melanocytes (Aubin-Houzelstein et al., 1998, Vance and Goding, 2004). Specification of melanoblasts from the neural crest is a process in which pluripotent cells become restricted to the melanocyte cell lineage and it is regulated by several signalling pathways including the crucial Wnt signalling pathway. Disruption of this pathway, using knockout mice, is linked to a dramatic reduction in the number of neural crest derived melanoblasts, while forced expression of Wnt signalling is linked to an increase in total melanoblast population (Ikeya et al., 1997, Dunn et al., 2000)

1.2.2 Melanoblast Differentiation & Migration

Melanoblasts are precursor pigment cells that have the melanocyte cell lineage specified but are immature and not yet pigmented. Beginning at the neural tube, melanoblasts migrate dorsally to ventrally during embryonic development and are found in the dermis, the epidermis and the hair follicle (Vance and Goding, 2004, Beauvais-Jouneau et al., 1999b). Once cell specification has been determined, melanoblasts migrate away from the neural tube, proliferating rapidly along specific migratory pathways (Cooper and Raible, 2009, Thomas and Erickson, 2008, Beauvais-Jouneau et al., 1999a). Melanoblasts migrate to specific sites

along the back and down either side of the body where they proliferate and spread down the legs and head and sides, eventually joining up under the neck and the belly.

In the skin, melanoblasts reside in the basement membrane of the epidermis, where, upon stimulation by the surrounding keratinocytes, they complete their differentiation into mature melanocytes, (Van Neste and Tobin, 2004, Straile, 1964, Krause and Foitzik, 2006). Following migration to the follicle, melanoblasts will either differentiate into mature melanocytes that pigment the initial fibre, or form a population of melanocyte stem cells that remain in reserve in the hair bulb to produce pigment for subsequent hair growth cycles (Nishimura et al., 2002, Van Neste and Tobin, 2004, Tobin and Bystryn, 1996)

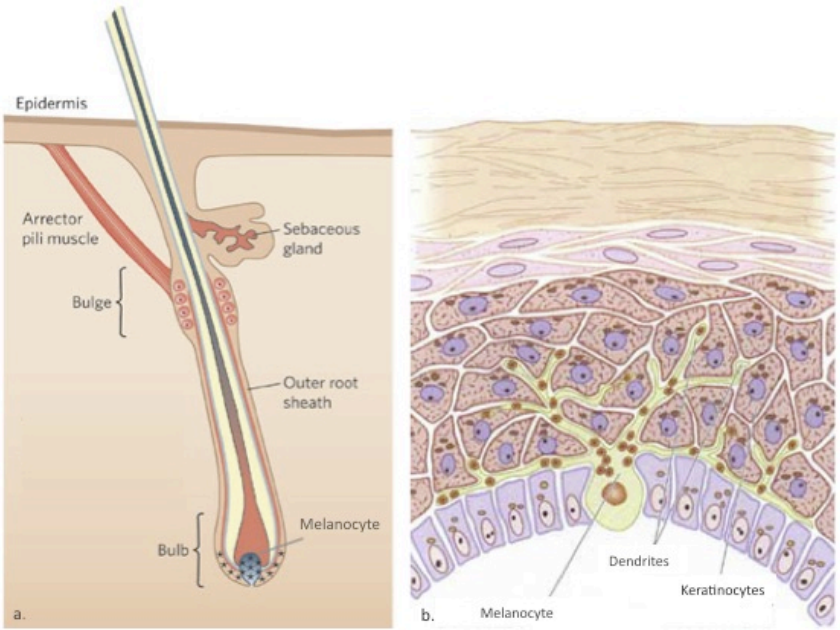


Figure 1.1 Localisation of melanocytes in the hair follicle (a) and skin (b). Melanocytes in hair reside in the bulb, from here pigment is incorporated into the growing follicle. In skin, melanocytes reside in the basal layer of the epidermis where they secrete mature melanocytes into the surrounding keratinocytes. Adapted from Lin and Fisher 2007.

1.2.3 Melanocytes and melanosomes

The structure of the melanocyte is a central cell body surrounded by long projections known as dendrites. The melanocyte is located in the basal layer of the epidermis and in the hair bulb where they secrete pigment into surrounding keratinocytes via the dendrites. Located within the cytoplasm of the mature melanocyte are specialized organelles called melanosomes, where pigment synthesis occurs (Hearing, 2000, Elleder and Borovansky, 2001, Seiji et al., 1961, Yuji and Vincent, 2009).

There are four stages of melanosome development, termed stages I to IV (Seiji et al., 1961). Stage I and II melanosomes lack pigment, but by stage II internal structures have begun to form, upon which pigment will be deposited. In late stage II and stage III melanosomes the catalytic enzymes that drive melanin synthesis are delivered, initiating melanin synthesis. By stage IV the fully pigmented, mature melanosome is ready to be transported to into surrounding cells, where it will be observed as visible pigment (Hearing, 2000, Scott, 2006, Lodish et al., 2007, Raposo and Marks, 2007). The melanocyte plays an important role, processing and transporting essential melanosomal proteins to specific regions within the cell during melanosome development.

1.2.4 Melanogenesis and Melanin

Several toxic by-products are produced as a consequence of melanin synthesis. Examples include hydrogen peroxide, superoxide and hydroxyl radicals, which are all known to cause oxidative stress (Land and Riley, 2000). These compounds are potentially damaging to DNA, which is thought to be one reason why melanin synthesis is confined to the melanosome. During the initial stages of melanogenesis, melanogenic enzymes are packaged in the Golgi and transported to the premelanosome. Once pigment synthesis is initiated pigment is deposited on internal structures within the developing melanosome and continues until the melanosome is completely filled. During the deposition of pigment the melanosome begins to move through the cytoplasm of the melanocyte towards the dendrites of the cell where, once completely filled with pigment, it will be transferred into the surrounding keratinocytes (Quevedo and Holstein, 1998, Scott, 2006, Schiaffino, 2010).

Melanin is responsible for the majority of colour phenotypes in mammals, affecting the colour of the skin, eyes and hair (Quevedo et al., 1985, Hearing and Tsukamoto, 1991, Pawelek and Korner, 1982). Variations the size, shape, type and distribution of melanin within skin and fibre are responsible for colour variation (Prota, 1992, Straile, 1964, Pawelek and Korner, 1982). In mammals, melanin is present in two forms, eumelanin and pheomelanin. Eumelanin is an oval shaped pigment, and pheomelanin is spherical and the larger of the two types at approximately 1μm in diameter as compared to approximately 0.7μm (Riley, 1997, Wasmeier et al., 2008, Ito, 1993). Most mammalian pigmentation is a result of a mixture of both pigment types, with the ration of eu- to pheomelanin determining the final colour (Hearing and Tsukamoto, 1991).

Chemical and enzymatic studies of melanins have provided useful insights into the distinct characteristics of the different melanin types (Prota, 1992) (Ito, 1986, Ozeki et al., 1995). Both melanin types are derived from a common precursor tyrosine, which undergoes oxidation by the tyrosinase enzyme, to form dopaquinone. Eumelanin synthesis is favoured by the activity of tyrosinase and an absence of thiol compounds. The addition of thiol compounds to dopaquinone, a highly reactive intermediate, results in the synthesis of 5-*S*-cysteinyldopa and *2*-*S*-cysteinyldopa which are further oxidised to form pheomelanin (Prota, 1992, Land and Riley, 2000, Furumura et al., 1998).

Eumelanin is a black/brown insoluble polymer that is derived from the oxidative polymerisation of 5,6-dihydroxyindol (DHI) and 5,6 dihydroxyindol-2-carboxylic acid (DHICA) (Kobayashi et al., 1994, García-Borrón et al., 2005, Jiménez-Cervantes et al., 1994). Eumelanin with a greater amount of DHI derived melanins leads to a black, insoluble, and high molecular weight pigment; while the presence of a higher proportion of DHICA-derived melanins results in a more soluble, lower molecular weight brown pigment (Figure 1.2) (Hearing and Tsukamoto, 1991, Kobayashi et al., 1994, Maeda et al., 1997).

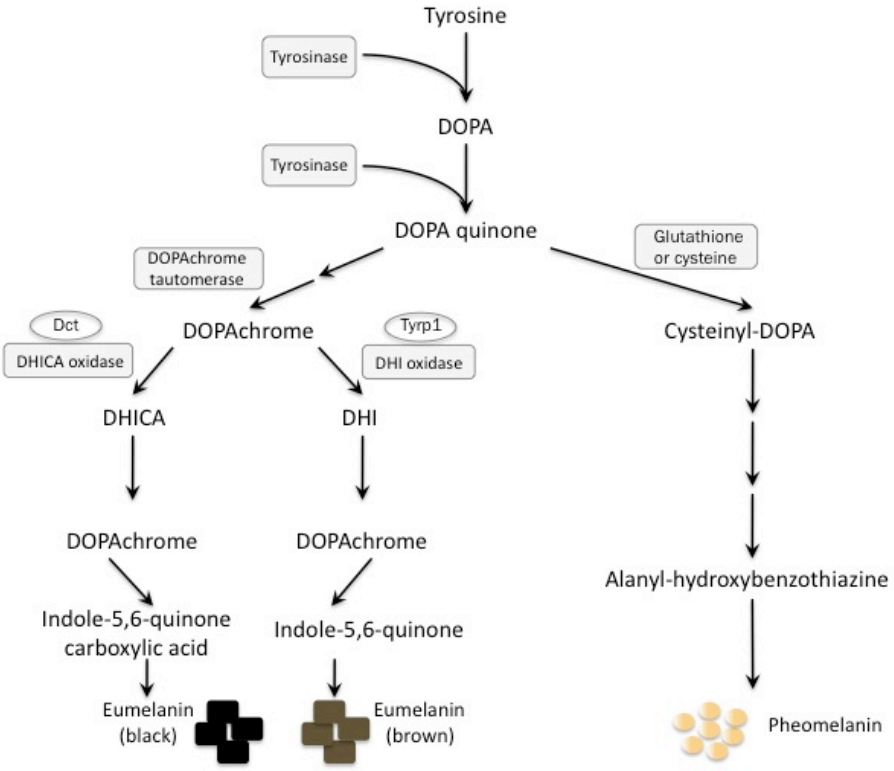


Figure. 1.2 Schematic Diagram of the pheomelanin and eumelanin synthesis pathways, adapted from Furumura et al. (1999). Both pathways share a common precursor, DOPAquinone. However, the pathways diverge based on activity of the melanocortin 1 receptor (MC1R) and its effect on the cellular environment.

Although the colours of mammals are caused by two pigments produced in a single pigment cell type (melanocytes), other species display different types of pigment cells. These pigment cells, known as chromatophores in general terms, serve the same basic function in all animals, but they differ in the type of pigments they produce. Fish, amphibians and some reptiles show more variability in the pigments types produced than do mammals, and also differ more widely in the functions of these pigments (Logan et al., 2006, Bagnara et al., 1979). Melanin is the predominant pigment type in mammals and birds, and is localized in the skin and hair/feathers (Hoekstra, 2006). However, pigment cells of many fish, amphibians and reptiles can disperse or aggregate to quickly to change the colour of an animal in response to hormonal or neuronal stimuli (Logan et al., 2006). This ability is often used as a means of camouflage or communication (Fujii, Nilsson et al., 2001). Fish, amphibians and reptiles also possess several

different types of pigment cells and are capable of producing more varied pigments. They include the melanophore/melanocyte, which produces melanin (black/brown), the xanthophore (red/yellow), iridophores (silver), cynaphores (blue) and leucophores (white) (Lamoreux et al., 2010).

1.2.5 Melanin export

The surface of the melanocyte contains long projections called dendrites that are interspersed among the surrounding keratinocytes (Berens et al., 2005, Minwalla et al., 2001, Scott et al., 2002). Investigations of human melanocytes have indicated that a single epidermal melanocyte can be connected to up to 40 keratinocytes (Quevedo and Holstein, 1998). This arrangement allows for the efficient transfer of mature melanosomes from the melanocyte into the neighbouring keratinocytes for incorporation into the skin or growing hair follicle (Hearing, 2000, Minwalla et al., 2001, Scott, 2006). Once the melanosome has matured and is fully pigmented it must be transported to the periphery of the cell, a function which depends on the interactions of cellular transport proteins including Rab27a, melanophilin and myosinVa (Provance et al., 2002, Wilson et al., 2000, Hume et al., 2002, Wu et al., 2006). These proteins form the link between melanosomes and the actin cytoskeleton that facilitates melanosome trafficking (Reviewed in Hammer and Wu, 2007). The basic process of melanin incorporation is the same for both skin and fibre, although the location of epidermal melanocytes and bulb melanocytes differs; with epidermal melanocytes located in the basal epidermal layer (Nascimento et al., 2003, Van Den Bossche et al., 2006) and bulb melanocytes residing in the hair bulb (Figure 1.1) (Tobin and Bystryn, 1996, Straile, 1964).

1.3 Regulation of melanogenesis

1.3.1 Pigment switching

A complex mechanism controls the switch between the types of melanin produced within the melanosome (Barsh et al., 2004, Rouzaud and Hearing, 2005, Le Pape et al., 2008). The switch is mediated by the actions of the membrane bound receptor Melanocortin 1 Receptor (MC1R). Through its interactions with alpha-

Melanocyte stimulating hormone (α-MSH) and Agouti signalling protein (ASIP) the receptor is able to modulate intracellular signals that stimulate pigment production acting as a biological on/off switch for pigment production (Oyehaug et al., 2002, Barsh et al., 2004).

Activation of the receptor occurs with binding of α-MSH and leads to eumelanogenesis. Binding of the α-MSH ligand to the receptor leads to a conformational change in the receptor that allows interaction with stimulatory guanine nucleotide-binding signal-transducing proteins (G proteins) within the cell which leads to the activation of adenylyl cyclase (Buggy, 1998). Adenyl cyclase is responsible for the formation of cyclic adenosine 3′, 5′-monophosphate (cAMP), an important secondary messenger which mediates trafficking of extracellular signals inside the cell (Tang and Gilman, 1992, Schramm and Selinger, 1984). In the melanocortin system, cAMP regulates the activity of downstream signalling processes that initiate melanogenesis (Busca, 2004, Garcia-Borron et al., 2005). High intracellular cAMP levels initiate an overall transcriptional upregulation of the cellular components necessary for pigment production including the melanosomal proteins TYR, Tyrp1 and Tyrp2 (Pajak et al., 1983, Cheli et al., 2009). These conditions lead the cell to initiate eumelanin production.

ASIP acts as an inverse agonist, competitively binding to MC1R and preventing α-MSH activation of the receptor (Barsh et al., 2004, Chai et al., 2003, McNulty et al., 2005, Sanchez et al., 2010). When ASIP is bound to the receptor, pheomelanogenesis occurs (Furumura et al., 1996, Sanchez-Mas et al., 2005a, Lu et al., 1994). When bound, ASIP blocks α-MSH binding to the receptor and also acts to stabilise the inactive receptor conformation (Sanchez-Mas et al., 2005a). The receptor is prevented from undergoing the conformational change necessary for G protein interactions and the resulting reduction in intracellular cAMP levels within the cell induces the production of pheomelanin.

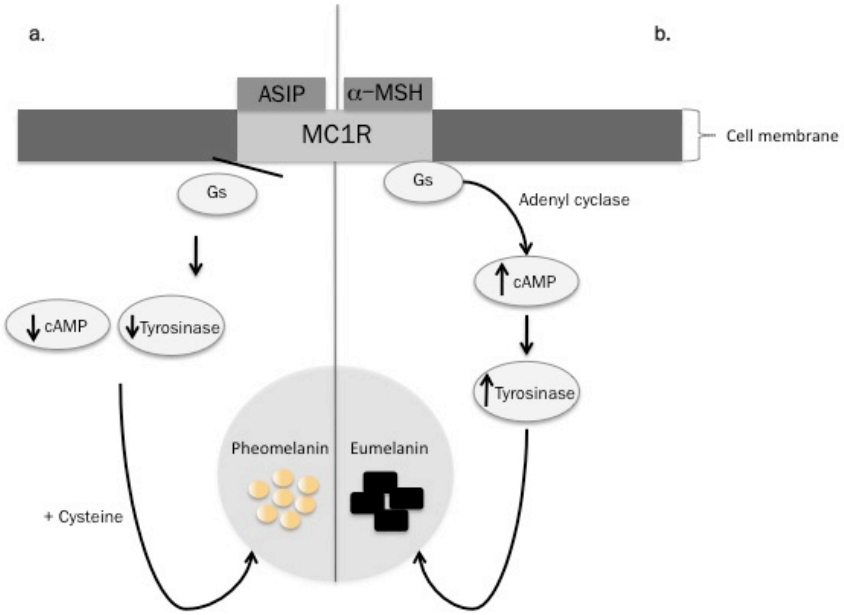


Figure 1.3. Schematic Diagram of MC1R controlled pigment production. a) Binding of the inverse agonist, Agouti signalling Protein (ASIP), inhibits receptor activation and leads to lower level of cAMP. These cellular conditions combined with the addition of thiol compounds, particularly cysteine, initiate pheomelanin production. b) Binding of α-Melanocyte Stimulating Hormone (α-MSH) to MC1R results in an activated receptor-ligand complex. This initiates transduction of a series of intracellular signals, including an increase of cAMP levels, leading to the formation of eumelanin.

1.3.2 MC1R

MC1R encodes a transmembrane protein expressed on the surface of melanocytes (Hoekstra, 2006, Rouzaud and Hearing, 2005, Mountjoy et al., 1992). This gene is known to play a key role in mammalian pigmentation and has been studied extensively, in part because of its small size and conserved structure (Smith et al., 2001, Butler and Cone, 2002, Kerje et al., 2003, Makova and Norton, 2005). *MC1R* consists of a single <1kb exon and is known to be highly polymorphic (Smith et al., 2001, Rana et al., 1999), with many well characterised allelic variants reported in mammals (Reviewed in Switonski et al., 2013, Jackson, 1997b, Majerus and Mundy, 2003). Previous investigations of the role of *MC1R* in mammalian pigmentation have confirmed *MC1R* to be one of the key genes controlling melanogenesis (Table 1.1).

Table 1.1 *MC1R* mutations in other species. Only mutations associated with fibre colour variation have been included and this list does not encompass all reported *MC1R* mutations.

Species	Polymorphism	Function significance	MC1R effect	Reference
Bear	A893G	Codon replacement	White Coat Colour	Ritland et al., 2001
Chicken	K92E	Activating mutation	Black Feathers	Takeuchi et al., 1996
Cow	L99P	Activating mutation	Dominant black	Klungland et al., 1995
Dog	C916T	Truncated protein, reduced receptor function	Light fibre	Everets et al., 2000
Dog	R301C	Reduced receptor function	Light fibre	Olliveret al., 2013
Horse	S83F	Non functional MC1R	Chestnut fibre	Markland et al., 1996
Human	A151C	Non functional MC1R	Red hair/light skin	Frandberg et al., 1998
Human	E269A	Reduced a-MSH binding	Red hair/light skin	Garcia-Borron et al., 2005
Human	T272A	Reduced a-MSH binding	Red hair/light skin	Garcia-Borron et al., 2006
Human	C273G	Loss of MC1R function	Red hair/light skin	Garcia-Borron et al., 2007
Human	R160W	Non functional MC1R	Red hair/light skin	Healy et al., 2001
Human	D294H	Non functional MC1R	Red hair/light skin	Healy et al., 2002
Human	T40I	Partial loss of function	Red hair/light skin	Jimenez-Cervantes et al., 2001
Human	V122M	Partial loss of function	Red hair/light skin	Jimenez-Cervantes et al., 2002
Human	D84E	Altered Cellular location	Red hair/light skin	Tully, 2007
Human	I155T	Altered Cellular location	Red hair/light skin	Tully, 2008
Human	V92M	Reduced a-MSH binding	Red hair/light skin	Tully, 2009
Human	R163Q	Reduced a-MSH binding	Red hair/light skin	Tully, 2010
Mammoth	R301S	Reduction in MC1R signalling	Light fibre	Rompler et al., 2006
Mouse	R65C	Reduced Receptor Function	Adaptive Beach Pattern	Hoekstra et al., 2006
Mouse	S69L	Activating Mutation	Black coat colour	Robbins et al., 1993
Mouse	E92K	Activating Mutation	Sombre phenotype	Robbins et al., 1993
Mouse	I98P	Cdon deletion	Yellow Coat Colour	Robbins et al., 1993
Mouse	W252C	Reduced Receptor Function	Tawny Coat Colour	Wada et al., 2005
Pig	D121N	Activating Mutation	Black coat colour	Kijas et al., 1998
Pig	A240T	Disrupts receptor function	Black coat colour	Kijas et al., 1998

Pig	L99P	Activating Mutation	Black coat colour	Kijas et al., 1998
Rabbit	304-333del	Reduced MC1R function	red/fawn/yellow	Fontanesi et al., 2006
Rabbit	280-285del	Effects second TM domain	White coat colour	Fontanesi et al., 2007
Sheep	M73K	Activating Mutation	Black coat colour	Vage et al., 1999
Sheep	D121N	Alters ligand binding	Black coat colour	Vage et al., 2000
Sheep	D121N	Activating mutation	Black coat colour	Yang et al., 2013
Sheep	M261K	Activating mutation	Black coat colour	Yang et al., 2013

MC1R is a member of the largest family of membrane proteins known as G protein coupled receptors (GPCR) (Yang, 2011, García-Borrón et al., 2005). There have been more than 900 unique GPCRs identified in humans, although the exact functions of a large proportion of these remain unknown (Schioth et al., 2005, Zhang et al., 2006, Zhou and Skolnick, 2012). GPCRs act as transducers for a range of extracellular signals, mediating a variety of physiological functions, including adrenal function (Sewer and Waterman, 2003, Tao, 2006), energy homeostasis (Carroll et al., 2005, Kristiansen, 2004), exocrine gland secretions (Ulloa-Aguirre et al., 1999, Schoneberg et al., 2004) and pigmentation (Sanchez-Mas et al., 2005a, Bockaert et al., 2002). GPCRs all display a common molecular structure consisting of an extracellular N-terminus, seven transmembrane domains connected by three extra- and three intracellular loops, and an intracellular C-terminus (Sanchez-Mas et al., 2005b, Schoneberg et al., 2004, Tao, 2006).

Among GPCR family members, the regions of the protein that are most conserved are the C-terminus and the portions of the transmembrane domain that are in close proximity to the cytoplasmic region. This conservation is most likely of functional significance as it is these regions that are involved in interactions with G proteins and subsequent activation of the adenyl cyclase pathway.

It is well established that *MC1R*, also known as the *Extension* locus, has an epistatic relationship to *ASIP* where a fully functioning MC1R is required for normal expression of ASIP alleles (Busca and Ballotti, 2000, Garcia-Borron et al., 2005, Hart, 2001).

1.3.3 ASIP

ASIP is the protein product of the *Agouti* gene and functions as an inverse agonist to MC1R (Furumura et al., 1996, McNulty et al., 2005, Ollmann et al., 1998). *ASIP* consists of three coding exons, which express a paracrine signalling molecule approximately 132 amino acids long (Bultman et al., 1992, Kwon et al., 1994, Vrieling et al., 1994). Similarly to MC1R, ASIP has several important structural features that are crucial for protein activity. These include a cysteine-rich carboxyl terminus, with 10 cysteine residues in which the spacing is conserved in all mammalian species, and a unique fold motif, known as an Inhibitor Cysteine Knot (ICK) (Yu and Millhauser, 2007). It has been shown that incorrect protein folding due to disruption of these features compromises the ability of ASIP to bind to MC1R, leading to loss of function phenotypes (McNulty et al., 2005, Yu and Millhauser, 2007).

ASIP also plays a role in pigment patterning in mammals by controlling the pattern of distribution of eumelanin versus pheomelanin throughout the skin and in each individual fibre of the animal. The wild type agouti phenotype (Fig 1.4.) in mice is characterised by hair with a sub-apical pheomelanic band on a eumelanic background (Kwon et al., 1994). The presence of both melanin types within a single hair suggests the presence of regulatory elements controlling hair-cycle specific activation of the gene. In mice, it has been established that the wild-type agouti phenotype is under the control of alternative hair-cycle specific promoters (Vrieling et al., 1994). However, this characteristic agouti pattern of pigmentation is not observed in all mammals.

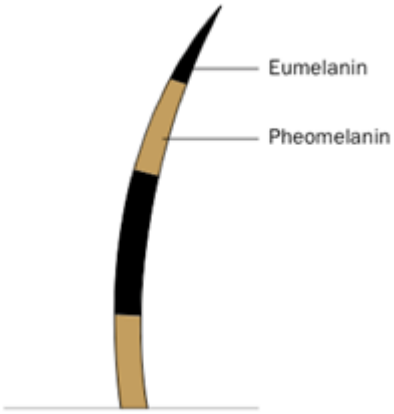


Fig. 1.4. Schematic diagram of the Agouti banded pattern on a single hair showing both eumelanin and pheomelanin pigments present in the same hair follicle. In mice, hair cycle specific promoters of ASIP control this phenotype. Adapted from Hoekstra 2006.

There is also evidence to suggest that there are regulatory elements of the Agouti gene responsible for region specific expression of the gene, for example the distinctive black and tan phenotype that is characterised by eumelanic pigment on the dorsal surfaces of the body and the lighter pheomelanic pigment confined to the ventral surfaces. This is an example of both pigments types being produced in the same animal concurrently but with tightly controlled boundaries. Homologous ASIP coding exons have been fully characterised in many species, but in some species alternative ASIP transcripts have been reported which arise from the presence of multiple alternative of non-coding exons (Vrieling et al., 1994, Vage et al., 1997, Kerns et al., 2004, Girardot et al., 2006, Fontanesi et al., 2010b, Girardot et al., 2004, Drogemuller et al., 2006).

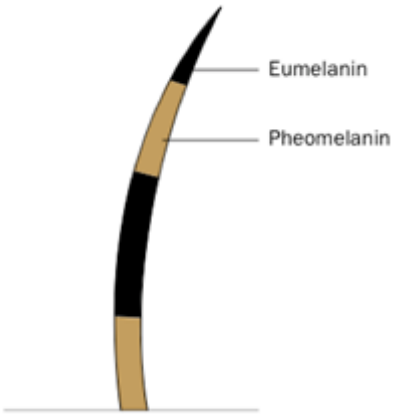
These unique regulatory elements have so far been identified in mice (Vrieling et al., 1994), rabbits (Fontanesi et al., 2010b), dogs (Kerns et al., 2004, Vage et al., 1997) pigs (Drogemuller et al., 2006) and cattle (Girardot et al., 2004, Girardot et al., 2006). In the mouse there are four non coding exons, two of which are confirmed to be associated with hair cycle specific expression and two with ventral specific expression (Miltenberger et al., 2002, Bultman et al., 1994). In the rabbit, alternative combinations of 5' untranslated region (UTR) sequences are associated with either dorsal or ventral expression of ASIP, which highlights the importance of the role of ASIP in pigment determination and patterning (Table 1.2) (Vrieling et al., 1994, Fontanesi et al., 2010b).

Table 1.2 Examples of *ASIP* mutations in other species that have been associated with fibre colour variation.

Species	Polymorphism	Functional significance	Agouti effect	Reference
Cat	2bp deletion in exon 2	Loss of function	Black phenotype	Eizirik et al., 2003
Dog	R96C	Non functional ASIP	Recessive black	Kerns et al., 2004
Dog	A82S	Unknown	Fawn-Sable phenotype	Berryere et al., 2005
Dog	R83H	Unknown	Fawn-Sable phenotype	Berryere et al., 2005
Fox	Deletion of exon 2	Loss of function	Black phenotype	Vage et al., 1997
Horse	11 bp deletion (Exon 2)	Frame shift mutation	Recessive black	Reider 2001
Rabbit	c.5_6insA (Exon 2)	Frame shift mutation, truncated protein	Black phenotype	Fontanesi et al., 2010
Sheep	Gene duplication	Alters gene expression	Black phenotype	Norris et al., 2008
Sheep	5bp deletion in exon 2	Loss of function	Black phenotype	Norris et al., 2008
Sheep	C126S	Loss of function	Black phenotype	Gratten et al., 2009

1.3.4 α-MSH

α-MSH is a small peptide hormone derived from the cleavage of its precursor hormone, pro-opiomelanocortin and which acts as an agonist to MC1R (Sturm et al., 2001, Hunt and Thody, 1995). Binding of α-MSH to MC1R mediates G protein interactions and the activation of the adenyl cyclase pathway. The subsequent rise in intracellular cAMP triggers a signalling cascade that initiates eumelanin synthesis (Bonetto et al., 2005, Sturm et al., 2001).



1.3.5 Enzymes that catalyse melanogenesis

Melanin synthesis, known as melanogenesis, is under the catalytic control of a group enzymes known as the Tyrosinase related protein family (TRP) (del Marmol and Beermann, 1996, Furumura et al., 1998). The TRP family includes tyrosinase (Tyr), tyrosinase related protein-1 (Tyrp1), and tyrosinase related protein-2, also known as DOPAchrome tautomerase (Tyrp2/Dct) (Budd and Jackson, 1995, Sturm et al., 1995). These proteins share significant structural homology and have enzymatic functions located within the melanosome (Box et al., 1998, Kobayashi and Hearing, 2007, Schmutz et al., 2002, del Marmol and Beermann, 1996).

Tyrosinase is the rate-limiting enzyme in melanogenesis, and its catalytic control of the melanin synthesis pathway is essential for the production of pigment (del Marmol and Beermann, 1996, del Marmol et al., 1993, Olivares et al., 2001). Animals without a functional tyrosinase enzyme are characterised by a complete absence of pigment and are associated with the albino phenotype (Tamate et al., 1985, Porter and Mintz, 1991, Rinchik et al., 1993, Beermann et al., 1995), because both eumelanin and pheomelanin are derived from a common precursor amino acid, tyrosine. While tyrosinase is responsible for catalysing the initial stages of melanogenesis, *Tyrp1* and *Dct* are also involved and act to modulate the quantity and quality of melanin produced (del Marmol and Beermann, 1996, Kobayashi et al., 1994),

Tyrp1 and *Dct* play a key role in melanin synthesis. Dct is responsible for the conversion of DOPAchrome to DHICA, rather than 5-6-dihydroxyindole (DHI) (Pawelek and Korner, 1982, Jiao et al., 2006, Kroumpouzos et al., 1994). *Dct* maps to the *slaty* locus in mice, and animals with mutations that affect the function of *Dct* show a diluted eumelanic phenotype. For example, the *slaty* phenotype has been shown to be a consequence of a higher proportion of DHI over DHICA derived melanins (Jackson et al., 1992, Budd and Jackson, 1995, Kroumpouzos et al., 1994).

Following the formation of DHICA intermediates, Tyrp1 activity promotes the oxidation of DHICA, resulting in the formation of final stage, black eumelanin

(Jiménez-Cervantes et al., 1994, Kobayashi et al., 1994). Melanin production may still occur without the activity of the Tyrp1 enzyme, however it leads to the production of a less stable intermediate polymer which is brown, rather than black (del Marmol et al., 1993, Kobayashi et al., 1994, Hearing and Tsukamoto, 1991, Jiménez-Cervantes et al., 1994). The first reported association between mouse *Tyrp1* and brown fibre colour was reported by Zdarsky et al. in 1990. In mice the wild type allele, *B,* produces black eumelanin while the recessive *b* allele produces a brown pigment. Since then several studies in mice have reported *Tyrp1* involvement in brown phenotypes (Shibahara et al., 1992, Sarangarajan et al., 2000, Lamoreux et al., 2001). Loss of function mutations in *Tyrp1* have also been linked with brown fibre colour in cattle (Berryere et al., 2003), cats (Lyons et al., 2004), canines (Schmutz et al., 2002), sheep (Gratten et al., 2007) and quail (Nadeua et al., 2007). Interestingly, DHICA oxidase activity is not a function of human Tyrp1. In humans, the tyrosinase gene performs this essential stage in melanin synthesis (Boissy et al., 1998, Olivares et al., 2001). Evidence suggests that the presence of *Tyrp1* in mammals is the result of a gene duplication of *Tyr*, which was then duplicated once more to give rise to *Tyrp2*. This resulted in two additional, highly similar proteins that share significant sequence homology, but have different enzymatic functions (Hearing and Tsukamoto, 1991, Jackson, 1994, Sturm et al., 1995).

1.4 Variations of melanogenesis

1.4.1 Common fibre colour phenotypes of mammals

The fibre/ hair/ fur colour of mammals is predominately due to the type and amount of melanin it contains. Melanin can be either black, brown or yellow/red with variations in the type, amount and distribution of melanin responsible for the wide variation in colour observed among mammals. Examples of common fibre colour phenotypes seen across mammalian species are shown in Figures 1.5-1.7. Black fibre colour is due to the presence of eumelanic pigment (Figure 1.5.).

Figure 1.5. Examples of black phenotypes in different species. All of these phenotypes are a result of the presence of eumelanic pigment. A. Black fibre colour seen in Labradors (Source http://cutedogsnpets.blogspot.com.au/2013/05/black-lablabrador-retrievers-pictures.html), b. A black fibre colour phenotype seen in horse (Sourcehttp://www.oocities.org/melissar91/wjimme.htm), c. Black mouse (Source http://www.tigm.org/) and d. A black fibre colour phenotype in the domestic cat (Sourcehttp://commons.wikimedia.org/wiki/File:Black_cat_at_Jacob's_Well_Nablus_005_Aug_2011.jpg).

The brown fibre phenotype is due to modifications during eumelanin synthesis that result in the production of brown eumelanin (Zdarsky et al., 1990, Shibahara et al., 1992). Brown eumelanin has been associated with the activity the *Tyrp1* gene in dogs (Schmutz et al., 2002), cats (Lyons et al., 2004, Schmidt-Küntzel et al., 2005), cattle (Berryere et al., 2003), mice (Shibahara et al., 1992, Zdarsky et al., 1990) and sheep (Gratten et al., 2007). Phenotypic descriptions of *Tyrp1* brown phenotypes in mammals vary between each species (Figure 1.6.). In cats *Tyrp1* mutations are associated with the chocolate and cinnamon phenotype (Lyons et al., 2004), the chocolate brown phenotype in dogs is caused by three documented Tyrp1 alleles (Schmutz et al., 2002) and mice have Tyrp1 alleles known as brown or cordovan (Orlow et al., 1993, Zdarsky et al., 1990).

Table 1.3 Reported *Tyrp1* mutations in species and their associated phenotypic effect

Species	Polymorphism	Functional significance	Tyrp1 phenotype	Reference
Cat	C298T	Premature stop codon in Exon 2	Cinnamon phenotype	Lyons et al., 2004
Cat	Donor splice site mutation in Exon 6	Addition of 17 amino acids	Chocolate phenotype	Schmidt-Kuntzel et al., 2005
Cat	A3G	Substitution in the signal peptide	Chocolate phenotype	Schmidt-Kuntzel et al., 2005
Cattle	H424Y	Unknown	Dun brown phenotype	Berryere et al., 2003
Dog	Q331X	Truncated protein	Brown phenotype	Schmutz et al., 2002
Dog	C41S	Interrupts formation of important disulphide bonds	Brown phenotype	Schmutz et al., 2002
Dog	345delP	Truncated protein	Brown phenotype	Schmutz et al., 2002
Human	368delA	Truncated protein which alters tyrosinase activity	OCA3-reduced pigment of skin hair and eyes	Boissy et al., 1996
Human	S166X	Truncated protein	OCA3 Brown albinism	Boissy et al., 1996
Human	C30R	Truncated protein, Incapable of melanin synthesis	OCA3 Brown albinism	Yamada et al., 2011
Japanese quail	F282S	Unknown	Roux (br^x) phenotype	Nadeau 2007
Mouse	Recessive mutation	Minimal expression of *Tyrp1* mRNA	Cordovan brown	Jackson et al., 1992
Mouse	Inversion in exons 1-3	Causes lightening of hairs near the body resulting in an overall pale brown appearance	White based brown phenotype	Javerzat et al., 1998
Mouse	C86Y	Incorrect trafficking of Tyrp1	Light phenotype	Orlow et al., 1993
Pig	c.1484_1489del	Truncated protein	Brown phenotype	Ren et al., 2011
Soay sheep	C290F	Loss of a conserved cysteine residue	Light phenotype	Gratten 2007

Although recessive *Tyrp1* brown has been proven to exist in a number of mammalian species there are some species where the *Tyrp1* brown phenotype does not exist, for example horses. It is also important to note when referring to Tyrp1 'brown' animals that these are animals that are brown at the level of pigment and are not animals that exhibit a phenotype that is visually interpreted as brown. It can often be difficult to visually differentiate between a genetic brown phenotype and some red phenotypes, which are caused by pheomelanic pigment.

Figure 1.6. Examples of eumelanic brown phenotypes in cats, dogs and mice. The examples given here are animals with documented mutations that cause brown eumelanic pigment, not animals that exhibit a phenotype that is visually interpreted as brown. a) Cinnamon cat (Source http://colorgenetics.info/feline/gallery/dilutions-andmodifiers/chocolate/cinna-mon-amour) b) Chocolate brown Labrador (Source http://www.thelabradorsite.com/?p=175) c) Chocolate brown cats (Source http://www.royalsapphire.cz/en_genetika.html) d) Brown mouse (Source http://www.espcr.org/micemut/locustyrp1.html)

Pheomelanic pigment results in fibre colours ranging from yellow to red (Ito and Fujita, 1985, Oyehaug et al., 2002, Le Pape et al., 2008). The chestnut colour in horses is due to a missense mutation of MC1R (S83F) which causes the exclusive production of pheomelanin (Markland et al., 1996) while a premature stop codon in MC1R is associated with the yellow coat colour in Labrador Retrievers (Schmutz et al., 2002) (Figure 1.7). Very dilute pheomelanin can appear white, while animals with strongly pigmented pheomelanic coats can appear red and are sometimes mistakenly described as brown, which is incorrect in terms of melanin type.

Figure 1.7. Examples of yellow/red fibre phenotypes in mammals. a. A chestnut horse, (Source http://www.myhorse.ca/horseoftheweek/2007/how20071008.htm) b. An orange rabbit (Source http://colorgenetics.info/leporine-rabbit/basic-rabbit-color-genetics-extension-and-agouti) c. A yellow Labrador Retriever dog, the result of a recessive MC1R allele (Source http://en.wikipedia.org/wiki/Labrador_Retriever) d. Red hair in humans, a non-functional variant of the MC1R gene (Source http://www.myredhairgene.com/)

Colour patterns are formed as a result of the distribution of the both pigment types in the one animal. A classic colour patterning phenotype example is the bay phenotype of horses, which is a result of differences in the distribution of black versus yellow pigment and is controlled by differential expression of the ASIP gene.

Melanogenesis is a highly complex and multi staged process with the potential for pigment to be affected at any stage (Prota, 1992, Kobayashi et al., 1995).

Pigmentary mutations can generally be classified into four main categories, based on the cellular processes they affect; these include melanocyte development and migration, melanosome development, melanosomal transport and pigment-type switching.

1.4.3 Defects of melanocyte development and migration

The differentiation, proliferation and migration of melanocytes is under the control of many genes, and these events are essential for pigmentation (Garcia-Castro and Bronner-Fraser, 1999, Wilkie et al., 2002). White spotting and age related greying are phenotypes that are caused by an interruption of melanocyte development and an absence of viable melanocytes, respectively (Wilkie et al., 2002, Vance and Goding, 2004).

The white spotting phenotype is present at birth and can range from localised white spotting to an entirely unpigmented animal (Lamoreux et al., 2010). White spotting is most common on the ventral body surfaces as this is the last region to receive migrating pigment cells (Beauvais-Jouneau et al., 1999a). White spotting that causes a completely unpigmented animal, or 'one big white spot', should not be confused with an albino phenotype, which is characterised by a complete absence of melanin. In white spotting the white fibre is a result of a lack of viable pigment cells, while albinism is a defect in the melanin synthesis pathway that prevents melanin production (Schmidt-Küntzel et al., 2005, Rinchik et al., 1993, Pawelek and Korner, 1982). Examples of genes linked to white spotting include *Mitf (Goding, 2000)* and *Ednrb (McCallion and Chakravarti, 2001)*, which control the extent of melanocyte migration and the proliferation and migration of melanoblasts respectively. Variations that affect activity of Mitf or Ednrb result in animals with a white chest, forehead and feet to varying degrees and have been reported as the cause of white spotting in both dogs and horses (Hauswirth et al., 2012, Baranowska Körberg et al., 2014).

Animals with progressive greying are pigmented at birth and show a progressive loss of viable melanocytes with age (Lamoreux et al., 2010, Schouwey et al., 2007, Goding, 2007). During normal regeneration of fibre follicles, differentiation of melanocyte stem cells is initiated to generate a new population of mature pigments cells ready for incorporation into the growing follicle (Nishimura et al.,

2002). Progressive greying is linked to genes that control the maintenance of melanoblasts and melanocyte stem cells, including Notch1 and Notch2, with the loss of pigment cells occurring during successive regeneration of fibre follicles (Schouwey et al., 2007).

1.4.4 Defects of melanosome development

Correct pigment synthesis requires a number of specialised enzymes (del Marmol and Beermann, 1996); structural proteins which form the melanosome matrix onto which pigment is deposited (Theos et al., 2005) and transporter proteins which control the level of intracellular constituents necessary for melanin synthesis (Di Pietro et al., 2006, Salazar et al., 2006). Altered functioning of any of these processes in the melanosome usually results in pigment variation across the entire animal, as the ability to synthesise pigment is compromised. Examples of these phenotypes include the albino phenotype (Figure 1.8) in which no pigment is produced due to a faulty tyrosinase enzyme (Rinchik et al., 1993, Hearing and Jimenez, 1987).

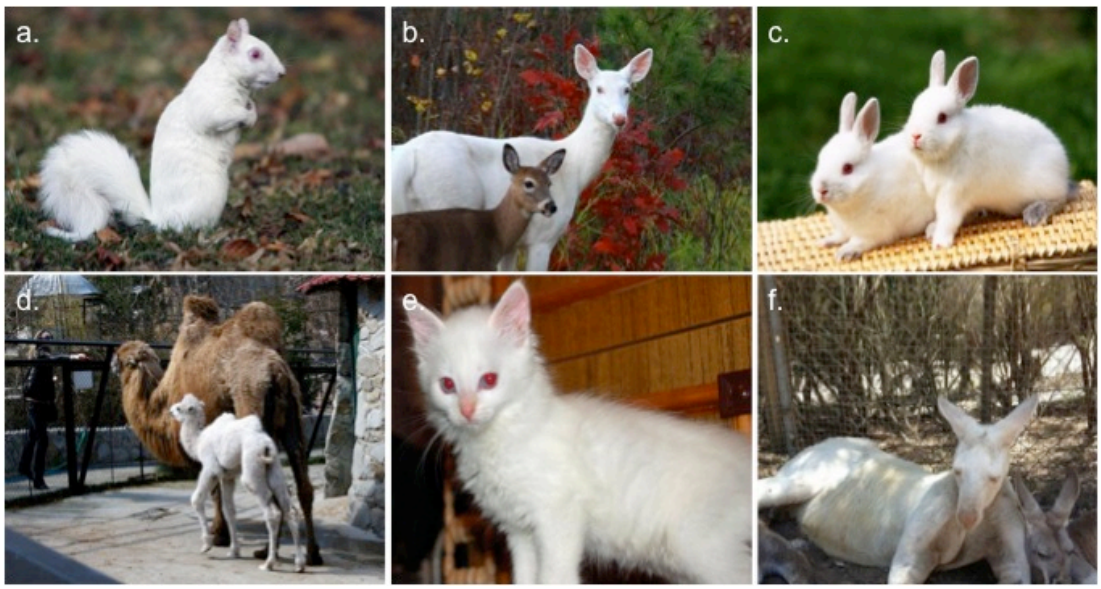


Figure 1.8. The albino phenotype in different mammalian species. a) Albino squirrel b) Albino deer c) Albino rabbits d) Albino camel e) Albino cat f) Albino Kangaroo (Source- http://www.telegraph.co.uk/earth/earthpicturegalleries/6338681/Albino-animals-from-Snowflake-the-white-gorilla-to-White-Diamond-the-alligator.html)

1.4.5 Defects of melanosome transport

Once melanin has been synthesised it must be exported out of the melanocyte via transfer to the dendrites and incorporation into the surrounding keratinocytes or growing hair follicle before it can be observed as visible pigment (Abdel-Malek et al., 2000, Elleder and Borovansky, 2001). There are several genes that have been linked to the transport of melanin; these include Melanophilin (Mlph), Myosin VA (MYO5A) and Ras-Related Protein Rab-27A (Rab27a) (Elleder and Borovansky, 2001, Provance et al., 2002). The protein products of these genes form a complex that assists in transporting the mature melanosomes to the periphery of the melanocyte where they can then be transferred to surrounding keratinocytes to provide pigment for skin and fibre (Van Gele et al., 2009, Provance et al., 2002). When any of these proteins function incorrectly or is not produced the ability to correctly traffic the melanosome is affected and instead of the normal distribution of pigment it results in clumping of the pigment granules, which appears as a diluted colour phenotype (Wilson et al., 2000, Hume et al., 2002, Ishida et al., 2006, Lane and Womack, 1979).

1.4.6 Defects of pigment switching

While melanocytes are capable of producing both pigment types, only one pigment is produced at any one time. A highly complex mechanism controls the switch between eumelanogenesis and pheomelanogenesis, mainly through the actions of the *MC1R*, *ASIP* AND α*-MSH* genes (Oyehaug et al., 2002, Barsh et al., 2004). Much of pigment patterning is attributed to the effects of the ASIP gene, switching on and off of ASIP expression during pigmentation results in animals with both pigment types present. ASIP expression can control the pigment type produced within a single hair as well as pigment types distributed across the entire animal, leading to colour pattern phenotypes such as black and tan where the animal displays eumelanic colouring on the dorsal aspect with a pheomelanic underbelly (Lu et al., 1994, Vrieling et al., 1994, Hustad et al., 1995).

The modulation of final colour phenotypes due to the eumelanin/phenomelanin switch mechanism is complex and is responsible for the large variation of fibre colours observed in mammals considering it is based on only producing two pigment types (Oyehaug et al., 2002, Le Pape et al., 2008, Kobayashi et al.,

1995). The ratio of eumelanin to phenomelanin is responsible for the final colour phenotype and it is under tight control by the interactions of MC1R and ASIP (Quevedo and Holstein, 1998, Hearing and Tsukamoto, 1991, Pawelek and Korner, 1982). Within the interaction of these 2 genes lies the possibility for both dominant and recessive eumelanic and dominant and recessive pheomelanic phenotypes. This is part of the reason why colour studies are complicated as both dominant black and recessive black alleles are phenotypically the same but under the control of different genetic loci.

Loss of function and gain of function polymorphisms are commonly reported in both the MC1R and ASIP genes in mammals that restrict pigment production to only one type. Examples where eumelanic fibre can either be caused by a dominant *MC1R* allele or the recessive *Agouti* allele include the D121N mutation that is a gain of function mutation in MC1R responsible for eumelanic fibre in pigs (Kijas et al., 1998) and the loss of function ASIP mutation, R96C in dogs, which is similarly associated with black fibre (Kerns et al., 2004). Pheomelanic fibre can be attributed to a recessive *MC1R* allele or a dominant *ASIP* gene respectively. Examples of these phenotypes include the loss of function MC1R variants responsible for chestnut horses (Marklund et al., 1996) and red hair in humans (Valverde et al., 1995, Schioth et al., 1999).

1.6 Proposed fibre colour series in alpacas

At the commencement of this study there was no universally accepted, well-supported theory that fully explained the mode of inheritance of white, colour and pattern phenotypes in alpacas. While previous theories attempted to explain the basic inheritance of colour in alpacas the majority have been based on breeding records and observed phenotypes, and no molecular investigations into alpaca colour genes had been reported.

Previously published alpaca fibre colour theories include the work by Sponenberg (2001), Hart (2001), and Paul (2005). In 2001 Sponenberg proposed an alpaca colour theory based on what was already known about the control of colour in related species including horse, dog, sheep and goats and what was observed

from alpaca breeding records. Sponenberg (2001) identified *MC1R* and *ASIP* as the likely determinants of the majority of colour variation. White was identified as the dominant phenotype, with black being recessive to all other colours. Sponenberg also questioned the existence of a true genetic brown phenotype in alpacas, stating that most browns phenotypes would likely be attributed to pheomelanic pigment.

Hart (2001) proposed a model for the inheritance of white fibre colour in alpacas and identified both *MC1R* and *ASIP* as having important interactions in colour determination. Hart reported a dominant white allele at the *ASIP* locus as well as at least two *MC1R* alleles, the wild type and one recessive allele.

Paul (2005) evaluated the proposed colour theories of both Hart and Sponenberg against breeding records from the Australian alpaca industry and indicated that while the models were able to explain some colour inheritance and mating outcomes they could not adequately explain all. For example, while Hart reported the existence of a dominant white allele in alpacas the breeding data for white animals examined by Paul did not support the presence of a large number of dominant white alpacas in the population.

These proposed models for fibre colour inheritance in alpacas were based on phenotype observations and breeding records. All authors agreed that there were limitations in what could be assumed because of inaccuracies in breeding records and previous practices of ignoring patterned areas of colour when assuming genotype. Sponenberg (2001) and Hart (2001) both recognised the importance of including patterns of colour when assuming genotype. Paul (2006) further expanded upon this assumption and highlighted the importance of taking into account all pigmented areas of the animal when assigning phenotype, including skin colour. Paul hypothesised that white animals with pink skin may be being controlled by different genes than white animals with black skin, and should therefore be treated differently when breeding for colour.

This project was directed at investigating the genetic mechanisms controlling fibre colour inheritance in alpacas with an emphasis on a phenotype classification

system that focused on the type of melanin present in the animal rather than a description of the visual colour. This project was focused specifically on the inheritance of the base coat colours black, white and brown.

1.6.1 Current strategies for predictive breeding and their limitations

The current strategies for predictive breeding in alpacas are based on the incomplete models of colour inheritance in alpacas and are based solely on the recorded phenotype for the animal. There are several reasons why these strategies are ineffective. Firstly, it is known that there is more than one genetic pathway to the same or similar phenotypes in mammals. For example a white animal may be recessive *MC1R* allele, a dominant *ASIP* allele or an extended white spotting allele. All these animals would look very similar but would have different outcomes when mated. Assumptions of genotype based on colour phenotype alone are therefore often likely to be incorrect.

Secondly, the lack of knowledge of the underlying genetics of fibre colour inheritance in alpacas has led to a high instance of incorrect phenotype assignment in alpaca breeding records (Paul, 2006). These breeding records are then used to make selections for animals to breed for particular colour phenotypes, leading to matings which produce offspring of unexpected colour phenotypes. This creates confusion and breeding records cannot be relied upon to support investigations of colour inheritance in alpacas.

Thirdly, phenotype assignment is very subjective and can be hard to assign visually (Cecchi et al., 2011). In addition, the common names used to describe phenotype in breeding records are often not based on scientific nomenclature or evidence. Colour phenotypes are often a visual description of colour that has no reflection on the causative pigment. There are several different methods used to classify colour including the Munsell colour system, natural fibre charts and more recently chemical analysis of melanin (Ozeki et al., 1996). Without a standard classification system it is difficult to consolidate the knowledge concerning colour inheritance in alpacas. This has highlighted the need for genetic research into pigmentation in alpacas so that breeders can make more informed decisions

when breeding for colour and to reduce the incidence of crias of unexpected or unwanted colour.

1.7 Aim and relevance of the study

The work described in this thesis has sought to characterize the major pigment genes involved in fibre colour determination in alpacas. This study employed a candidate gene approach to investigate three fibre colour genes, *MC1R*, *ASIP* and *Tyrp1*, which are known to be responsible for the three base colour phenotypes, yellow/red, black and brown in mammals. The major aims of this study were to:

1) Characterise the *MC1R*, *ASIP* and *Tyrp1 genes in alpaca*

2) Determine the relationships between polymorphisms in these pigment genes and the phenotypic variation observed in Australian alpaca populations

3) Determine if eumelanic brown exists in alpacas and investigate if *Tyrp1* is the cause of the 'visual brown' phenotype observed in alpacas

2

General Materials and Methods

This chapter details the general laboratory reagents and methods used throughout the project. Laboratory resources and methods specific to individual experiments are described in their respective chapters.

2.1 Alpaca samples

All research was conducted with Curtin University Animal Ethics approval (043-2008, R01/2009, R06-10 and AEC_2011_23). This study focused on a variety of fibre colour phenotypes in alpacas: white, fawn, chestnut, brown and black animals thought to be primarily under the control of the three genes examined in this study (preference was given to solid coloured animals, i.e. unpatterned). In order to avoid sampling/environmental bias, alpaca DNA samples were sourced from 10 Alpaca breeders in Western Australia, Victoria and New South Wales who consented to the inclusion of their animals in this project. Both male and female alpacas were sampled in both the Suri and Huacaya fibre types. Owners were required to provide information to uniquely identify each animal, as well as the relationship of the animal to any other in the study.

Blood and fibre samples were collected from each animal and digital photographs were taken for phenotype recording (Table 2.1). Approximately 5ml of blood was collected from the jugular using a 21 or 23 gauge needle. Blood was collected into 5ml K_2EDTA tubes and was kept at 4°C until DNA extraction was performed (<3 days). Remaining blood samples and extracted DNA was stored at -20°C. Fibre samples were collected from the body, and any other areas of differing colour. Approximately 200mg of fibre was collected from each animal and stored in airtight plastic bags at room temperature. Digital photographs were taken of each animal (where possible) of the head, body and close-ups recording the skin colour around the eyes and feet.

Table 2.1. Fibre colour phenotypes of animals included in this study, ✓ indicates the chapters where they were included in the sample cohort for analysis. X indicates that the analysis was not performed for that gene. * indicates skin colour was not determinable for the animal.

Sample ID	Fibre Colour	Skin Colour	MC1R	Agouti	TYRP1
ALP001	Fawn	Black	✓	✓	×
ALP002	Bay	Black	✓	×	×
ALP003	Rosegrey	Light	✓	×	×
ALP004	Bay	Black	✓	×	×
ALP005	White	Black	✓	✓	×
ALP006	White	Light	✓	✓	×
ALP007	Bay	Black	✓	×	×
ALP008	Fawn	Black	✓	×	×
ALP009	White	Light	✓	✓	×
ALP010	Fawn	Black	✓	×	×
ALP011	Fawn	Black	✓	✓	×
ALP012	Black	Black	✓	✓	×
ALP013	Black	Black	✓	×	×
ALP014	White	Light	✓	✓	×
ALP015	Black	Black	✓	✓	×
ALP016	Fawn	Light	✓	×	×
ALP017	Bay	Black	✓	×	×
ALP018	White	Light	✓	✓	×
ALP019	White	Light	✓	✓	×
ALP020	White	Light	✓	✓	×
ALP021	White	Light	✓	✓	×
ALP022	White	Light	✓	×	×
ALP023	Silvergrey	Black	✓	×	×
ALP024	White	Black	✓	✓	×
ALP025	Bay	Black	✓	×	×
ALP026	White	Black	✓	✓	×
ALP027	Bay	Black	✓	×	×
ALP028	Black	Black	×	✓	×
ALP029	White	Light	×	✓	×
ALP030	Light Fawn	Light	×	✓	×
ALP031	Black	Black	✓	✓	×
ALP032	Black	Black	✓	✓	×
ALP033	Brown	Black	×	×	✓
ALP034	Light Fawn	*	✓	×	×
ALP035	Black	Black	✓	✓	×
ALP036	Black	Black	✓	✓	×
ALP037	Black	Black	✓	×	×
ALP038	Black	Black	✓	✓	×
ALP039	Black/Brown	Black	✓	✓	×
ALP040	Black/Brown	Black	×	✓	×
ALP041	Black/Brown	Black	×	✓	×
ALP042	Black	Black	×	✓	×

ALP043	Black	Black	×	✓	×
ALP044	Black	Black	×	✓	×
ALP045	Black	Black	×	✓	×
ALP046	Black	Black	×	-	×
ALP047	White	Black	×	✓	×
ALP048	White	Black	×	✓	×
ALP049	Black	Black	✓	✓	×
ALP050	Black	Black	×	✓	×
ALP051	Black	Black	×	✓	×
ALP052	Black	Black	×	✓	×
ALP053	Black	Black	×	✓	×
ALP054	Black	Black	×	✓	×
ALP055	Black	Black	×	✓	×
ALP056	Black	Black	×	✓	×
ALP057	Black	Black	×	✓	×
ALP058	Black	Black	×	✓	×
ALP059	Black	Black	×	✓	×
ALP060	Brown	Black	×	×	✓
ALP061	Brown	Black	×	✓	✓
ALP062	Black	Black	✓	✓	×
ALP063	Black	Black	×	✓	×
ALP064	Black	Black	×	✓	×
ALP065	Black	Black	×	✓	×
ALP066	Black	Black	×	✓	×
ALP067	Dark Brown	Black	×		✓
ALP068	Black	Black	×	✓	×
ALP069	Black	Black	×	✓	×
ALP070	Black	Black	×	✓	×
ALP071	Black	Black	×	✓	×
ALP072	Dark Brown	Black	×	✓	✓
ALP073	Black	Black	×	✓	×
ALP074	Dark Brown	Black	×	✓	✓
ALP075	Black	Black	×	✓	×
ALP076	Fawn	Black	✓	×	×
ALP077	Fawn	Black	×	✓	×
ALP078	Fawn	Black	×	✓	×
ALP079	Fawn	Light	×	✓	×
ALP080	White	Light	×	×	✓
ALP081	White	Light	×	×	✓
ALP082	Light Fawn	Black	×	×	×
ALP083	White	Black	×	✓	×
ALP084	White	Black	×	✓	×
ALP085	White	Black	×	×	✓
ALP086	White	Black	×	×	✓
ALP087	Fawn	Light	×	✓	×
ALP088	White	Light	×	✓	×
ALP089	White	Light	×	×	✓
ALP090	Black	Black	×	✓	✓
ALP091	Black & Tan	Black	×	✓	×
ALP092	Dark Brown	Black	×	✓	×
ALP093	Dark Brown	Black	×	✓	✓

ALP094	Black	Black	×	✓	✓
ALP095	Black	Black	×	✓	×
ALP096	Dark Brown	Black	×	✓	✓
ALP097	Dark Brown	Black	×	×	✓
ALP098	Black	Black	×	✓	✓
ALP099	Brown	Black	×	×	✓
ALP100	Fawn	Light	×	✓	×
ALP101	White	Light	×	✓	×
ALP102	White	Light	✓	×	×
ALP103	Fawn	Light	✓	×	×
ALP104	White	Light	✓	×	×
ALP105	Black	Black	✓	✓	×
ALP106	Black	Black	✓	✓	×
ALP107	Brown	Black	×	✓	✓
ALP108	Brown	Black	×	×	✓
ALP109	Black	Black	×	✓	✓
ALP110	Black/Brown	Black	×	✓	×
ALP111	Black/Brown	Black	×	✓	×
ALP112	Black	Black	✓	✓	×
ALP113	Black	Black	×	✓	×
ALP114	Black	Black	×	✓	×
ALP115	Black	Black	✓	✓	×
ALP116	Black	Black	×	✓	×
ALP117	Black	Black	×	✓	×
ALP118	Black	Black	×	✓	×
ALP119	Warm Brown	Black	×	×	✓
ALP120	Red Brown	Black	×	×	✓
ALP121	Red Brown	Black	×	×	✓
ALP122	Dark Brown	Black	×	×	✓
ALP123	Warm Brown	Black	×	×	✓
ALP124	Dark Brown	Black	×	×	✓
ALP125	Dark Brown	Black	×	×	✓
ALP126	Dark Brown	Black	×	×	✓
ALP127	Black	Black	✓	✓	×
ALP128	Black	Black	✓	✓	×
ALP129	Black	Black	✓	✓	×
ALP130	Black	Black	✓	✓	×
ALP131	Black	Black	✓	✓	×
ALP132	Dark Brown	Black	✓	✓	×
ALP133	Fawn	Black	✓	×	×
ALP134	Silvergrey	*	✓	✓	×
ALP135	White	Light	✓	✓	×
ALP136	Chestnut	Light	✓	×	×
ALP137	White	Light	✓	×	×
ALP138	White	Light	×	×	✓
ALP139	White	Light	×	×	✓
ALP140	White	Light	×	×	✓
ALP141	White	Light	×	×	✓
ALP142	White	Light	×	×	✓
ALP143	White	Light	×	×	✓
ALP144	Black	Black	×	×	✓

ALP145	Black	Black	×	×	✓
ALP146	Black	Black	×	×	✓
ALP147	Black	Black	×	×	✓
ALP148	Black	Black	×	×	✓
ALP149	Black	Black	×	×	✓
ALP150	Warm Brown	Black	×	×	✓
ALP151	Warm Brown	Black	×	×	✓
ALP152	Warm Brown	Black	×	×	✓
ALP153	Dark Brown	Black	×	×	✓
ALP154	Black	Black	×	×	✓
ALP155	Black	Black	×	×	✓

2.2 Phenotype descriptions

There is no standardised colour nomenclature in alpacas, so for the purposes of this study animals were assigned to one of 17 colour groups based on adherence to the guidelines shown in table 2.2. These guidelines form the basis for the phenotype assignment criteria published by Cransberg *et al*. (2013) and are focused on the predominant melanin type observed rather that assigning colour descriptions, which can be subjective. Breeder descriptions of colour often have no relationship to genotype, which may negatively impact analysis.

Table 2.2. Phenotypic descriptions used to assign animal samples to a colour group. Adapted from Cransberg *et al.* (2013).

Phenotype description	Colour Phenotype
Amelanic (or extremely weakly melanic) fibre with pheomelanic skin	White (Pink-skinned)
Amelanic (or extremely weakly melanic) fibre with eumelanic skin	White (Dark-skinned)
Low but visibly detectable amounts of pheomelanin, with pheomelanic skin	Light Fawn (Pink-skinned)
Low but visibly detectable amounts of pheomelanin, with eumelanic skin	Light Fawn (Dark-skinned)

Moderate amounts of pheomelanin, with pheomelanic skin	Fawn (Pink-skinned)
Moderate amounts of pheomelanin, with eumelanic skin	Fawn (Dark-skinned)
Pheomelanic fibre and skin	Chestnut
Eumelanic skin, black face & extremities pale mixed melanin body fibre	Light Bay
Eumelanic skin, black face & extremities moderate intensity mixed melanin body fibre	Bay
Eumelanic skin, black face & extremities dark mixed melanin body fibre	Dark Bay
Eumelanic skin, black face & extremities, bright, intense mixed melanin body fibre	Bay
Eumelanic skin, extremely dark mixed melanin body fibre (may be difficult to distinguish from black by eye)	Black Brown
Eumelanic skin, eumelanic body fibre with ventral, jaw and/or extremity pheomelanic fibre	Black & Tan
Pheomelanic or mixed pheomelanic- eumelanic fibre, diluted over whole body, with or without non-diluted patches	Rosegrey
Amelanic fibres mixed with coloured fibres (pheomelanic or eumelanic), proportion of amelanic fibres increases over time	Roan
Eumelanic skin and fibre	Black
Eumelanic fibre, diluted differentially over whole body, with or without non-diluted patches	Silvergrey

2.3 DNA extraction and purification

2.3.1 DNA extraction from blood

Genomic DNA was extracted from 200 µl of EDTA anticoagulated alpaca blood using the Axyprep Blood Genomic DNA Miniprep Kit (Axygen), as per the manufacturer's protocol. The quantity and quality of DNA extracted was measured by electrophoresis on agarose gels (1% w/v) in 1% Tris-Acetic EDTA ph8 (TAE) and spectrophotometrically using a Nanodrop 1000 (Thermo Scientific). DNA was stored at -20°C.

2.3.2 Purification of DNA amplicons from polymerase chain reaction (PCR)

PCR products were purified using the Axyprep PCR Cleanup Kit (Axygen) according to the manufacturer's standard protocol. In order to ensure that PCR induced errors were minimised for subsequent sequencing, prior to clean up, five identical, independent 10 µl PCRs were pooled and adjusted to a pH of 6.5. Elution buffer (30 µl) was used to elute amplified DNA from the column.

2.3.3 Purification of DNA products

Ethanol was used to precipitate DNA following laboratory modifications such as restriction enzyme (RE) digestion or ligation. Two and a half times sample volume of 100% ethanol and 0.1 × volume of 3 M sodium acetate (pH 5.2) were added to the DNA solution and incubated at -20°C for 18 hrs. Samples were then centrifuged at 17,500g (4°C) for 30 minutes. The supernatant was removed and the pellet washed with 2 × volume of 70% ethanol and centrifuged at 17,500g for 15 minutes. The supernatant was removed and the pellet air-dried at room temperature for 15 mins and resuspended in Tris-EDTA (TE) buffer.

2.3.4 Purification of plasmid DNA

A QIAprep® Spin Miniprep Kit (Qiagen) was used for the extraction of plasmid DNA from ELECTROMAX™ DH5α-E™ *Escherichia coli*. A single bacterial colony containing plasmid was inoculated into 10 ml of Luria Bertani (LB) broth culture media containing ampicillin (100 µg/ml). The culture was incubated at 37°C for 18 hours with constant agitation. Bacteria were harvested by centrifugation at 2,600g for 15 minutes. Plasmid DNA was extracted as per the manufacturer's instructions, with the exception that plasmid DNA was incubated at room temperature with Tris.HCl (pH 8.0) for 1 minute prior to the final elution of DNA.

2.4 Sub-cloning of DNA fragments into a plasmid vector

2.4.1 pGEM Vector

The sub-cloning of difficult templates for sequencing and subsequent analysis was performed using the pGEM®-T Easy Vector System (Promega).

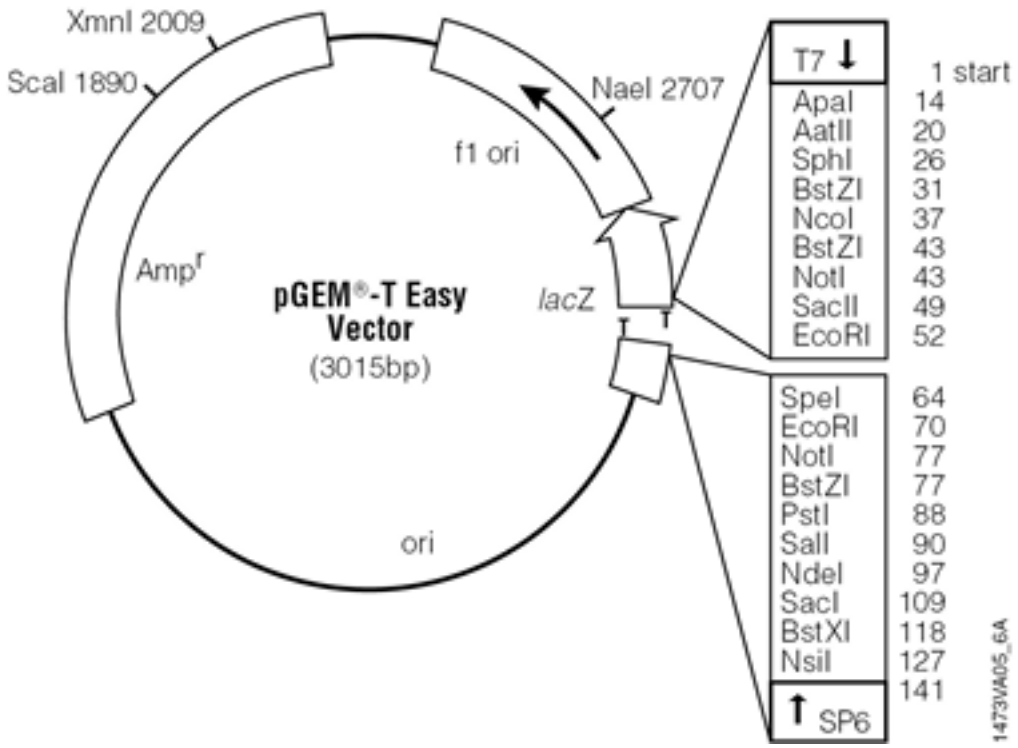


Figure 2.1. A map of pGEM®-T Easy Vector showing the location of restriction enzyme active sites and universal primers. (Source https://au.promega.com/~/media/files/resources/protcards/pgem%20t%20and%20pgem%20t%20easy%20vector%20systems%20quick%20protocol.pdf).

2.4.2 DNA ligation

Ligation of purified PCR amplicons was performed in 10 µl reactions comprising 50 ng of pGEM®-T Easy Vector DNA, insert DNA (optimised for a 1:1 insert: vector ratio), Rapid Ligation buffer (30 mM Tris.HCl, 10 mM MgCl2, 10 mM DTT, 1 mM ATP pH 7.8) and 3 units of T4 DNA ligase (Promega), which were incubated at 4°C for 18 hrs. The ligated products were purified as described in Section 2.3.3 and were resuspended in 5 µl of hpH_2O.

2.4.3 Transformation

A 1-µl aliquot of the purified ligation mix (Section 2.4.2) was mixed with 20 µl of ELECTROMAX™ DH5α-E™ *Escherichia coli* cells and transferred into an ice-cold electroporation cuvette (1mm). Electroporation was performed using the BioRad GenePulse II at the following settings; 1.8 kilovolts, 25 µF capacitance, and 200 to 500 ohms resistance range. The transformation mix was then immediately mixed with 700 µl of Super Optimal broth with Catabolite repression medium (SOC; Invitrogen) and incubated at 37°C for 60 minutes with gentle agitation. An aliquot of the culture (100 µl) was spread onto an agar plate [isopropyl β-D-1 thiogalactopyranoside (IPTG; 50 µg/ml), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; 50 µg/ml), ampicillin (100 µg/ml) Luria Bertani broth with Agar (LBA)] and were incubated at 37°C for 16-18 hours.

2.4.4 Selection of clones

Transformants containing insert DNA were identified using one of two methods:
i. Plasmid DNA extraction, followed by gel electrophoresis. Selected white colonies were grown in 6 ml of LB broth containing ampicillin (100 µg/ml) in preparation for plasmid DNA extraction as per Section 2.3.4. Recombinant plasmid DNA was identified by size comparison to a DNA molecule marker (Axygen) after electrophoresis in a 1.5% w/v TAE agarose gel.

ii. By PCR amplification of insert DNA in the plasmid using M13 forward and M13 reverse primers, followed by gel electrophoresis. PCR was carried out for each selected white colony in a 10-µl reaction including 1 µl of diluted bacterial culture (1 µl of one bacterial colony suspended in 10 µl of sterile hpH_2O). Standard PCR reaction mix and PCR cycling conditions were applied (Section 2.5.3). Positive amplicons were identified by size comparison to a DNA marker (Axygen) on a 1.5% w/v TAE agarose gel.

2.5 DNA amplification by PCR

2.5.1 Primer design

For genomic regions where sequence information was available, alpaca genomic DNA was amplified using specifically designed oligonucleotide primers for the *MC1R*, *ASIP* and *Tyrp1* genes. These primers were designed to hybridise at least

100bp outside of the predicted splice sites for each exon, thereby amplifying the complete coding region for each gene. Primer design was based on the alpaca genome assembly available on the Ensembl database (http://www.ensembl.org/index.html).

For genomic regions where alpaca sequence was not available, the Primer 3 program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to design primers that hybridised to conserved regions of the aligned bovine (*Bos taurus* GenBank accession number: NM_174108) and mouse (*Mus musculus*) sequence (GenBank accession number: NM_008559). Annealing temperatures (Tm) were determined using primer analysis software, NetPrimer (http://www.premierbiosoft.com/netprimer/) and in-house optimisation.

Table 2.3. Oligonucleotide primer sequences for amplification of alpaca exons.

Chapter	Gene	Primer Name	Sequence	Amplicon Size	Tm
3	*MC1R*	MC1R1-F	5' CTGGCACCACGGAGCTTG 3'	1371bp	58°C
3	*MC1R*	MC1R2-R	5' CTCCTCAGCCTGCTCCATTC 3'	1367bp**	
3	*MC1R*	MC1R3-F	5' GGGAGAAGGTGAGTGTGAGG 3'	1232bp	58°C
3	*MC1R*	MC1R4-R	5' GCTCTTCCTGGAGATTCGTG 3'	1228bp**	
3	*MC1R*	MC1R5-F	5' CATGGTGTCCAGCCTCTGCT 3'	Seq*	60°C
3	*MC1R*	MC1R6-R	5'CCACCCAGATGGCCGCGATG 3'	Seq*	60°C
3 & 5	*Universal*	M13-F	5'CCCCAGGGTTTTCCCAGTCACGAC 3'	NA	60°C
3 & 5	*Universal*	M13-R	5'TCACACAGGAAACAGCTATGAC3'	NA	60°C
4	*Universal*	T7-F	5' TAATACGACTCACTATAGGG 3'	Seq*	60°C
4	*Universal*	BGH-R	5' TAGAAGGCACAGTCGAGG 3'	Seq*	60°C
5	*Tyrp1*	Ex2F	5'CGGCTACATGGATTGACTTCC 3'	850 bp	60°C
5	*Tyrp1*	Ex2R	5' TTCACTTTGAGGTGGGTTGG 3'		
5	*Tyrp1*	Ex3F	5' TGAAATTGCTTGGTCAGTGC 3'	520 bp	61°C
5	*Tyrp1*	Ex3R	5' GCTCATCTCTACCCACGCTC 3'		
5	*Tyrp1*	Ex4F	5' GAAGTGTTTCCCAGCAAGG 3'	2503 bp	65°C
5	*Tyrp1*	Ex4R	5' TTTGACCTGGGAGTTCTC 3'		
5	*Tyrp1*	Ex4F2	5' CCTCTGTAGTCTGTAGTCAT 3'	Seq*	60°C
5	*Tyrp1*	Ex4R2	5' AAGAGTTTGGGATTGGCAGA 3'	Seq*	60°C
5	*Tyrp1*	Ex4R3	5' TCCAGTGATCTGAGTGCCAC 3'	Seq*	60°C
5	*Tyrp1*	Ex4F3	5'AACGTTTATTCTGCGTATGTTTTT 3'	Seq*	60°C

5	*Tyrp1*	Ex4F4	5' GCTTCACACCAAAACCCACT 3'	Seq*	60°C
5	*Tyrp1*	Ex4IF	5' AAGCCAAGCAAAGGGAGAAT 3'	560 bp	62°C
5	*Tyrp1*	Ex4IR	5' GCTTCACACCAAAACCCAC 3'		
5	*Tyrp1*	Ex5F	5' CCACATTACCTCAGGCAAGC 3'	2500 bp	62°C
5	*Tyrp1*	Ex5R	5' ATGACCAGTGATGGGAGA 3'		
5	*Tyrp1*	Ex5IF	5'AATCACAGAAGTTGGACATGG3'	Seq*	60°C
5	*Tyrp1*	Ex5IR	5' ATCAATCTGGCATTCAAAGGT 3'	Seq*	60°C
5	*Tyrp1*	Ex6F	5' TGTTGAGCCTGCAAAAA 3'	304 bp	55°C
5	*Tyrp1*	Ex6R	5'TGTTTCCCAATATCATCACTGT 3'		
5	*Tyrp1*	Ex7F	5' TTTTGGGTCACCTTCAGAACA 3'	287 bp	57°C
5	*Tyrp1*	Ex7R	5'GGGTAACACATTTGCTTTTGG 3'		
5	*Tyrp1*	Ex8F	5' TTTGCTCTCATTTCCTTTTTCA 3'	306 bp	57°C
5	*Tyrp1*	Ex8R	5'AGCTTTTAATTCCAACCTGTGC 3'		
6	*ASIP*	Ex2F	5' CTCAACTGGGACACTTGTGG 3'	416bp	60°C
6	*ASIP*	Ex2R	5' AGCACAAAGGAGCTGTGACC 3'		
6	*ASIP*	Ex3F	5' TCTATTCAGCCAACCCTTCG 3'	350bp	60°C
6	*ASIP*	Ex3R	5' GGTCTGGTCAGAGCTCAAGG 3'		
6	*ASIP*	Ex4F	5' TAAGTCCGAGCAGGTAGTGG 3'	570bp 627bp***	60°C
6	*ASIP*	Ex4R	5' AGGGAGCATGTGCGTAGC 3'		

*Primers used for sequencing reactions only

**Dependent on presence or absence of a 4bp deletion (c.225_255del)

***Dependent on presence or absence of a 57bp deletion (c. 325_381del).

2.5.2 Primer optimisation

Each primer set was optimised using a gradient PCR for annealing temperature and magnesium concentration. A 10°C temperature gradient and three magnesium concentrations (1mM, 1.5mM, 2mM) were tested to determine optimum conditions.

2.5.3 Amplification of DNA by PCR

Alpaca genomic DNA was amplified using the previously described primers (Section 2.5.1). The standard composition of the PCR master mix was as follows: 67mmol/l Tris.HCl (pH 8·8), 16·6 mmol/l $[NH_4]_2SO_4$, 0·45% (v/v) Triton X-100, 0·2mg/ml gelatin, 0·2 mmol/l dNTP (Fisher Biotec), 2μM each of forward and reverse primer, 1 unit Taq DNA polymerase (Fisher Biotec), 1·5 mM $MgCl_2$ and approximately 50-100 ng genomic DNA. Thermal cycles were: initial denaturation at 95°C for 3 min, followed by 30 cycles, each consisting of 94°C for 30s,

annealing temperature for 30 s and 72°C for 1 min/kb; with a final extension at 72°C for 10min. All PCRs were carried out in an Eppendorf Mastercycler (Eppendorf), in 10µl reactions. Any modifications from the standard PCR conditions are discussed in the relevant chapters.

2.5.4 Gel electrophoresis

Quality and quantity of the amplified DNA was determined by agarose gel electrophoresis. Electrophoresis was performed at 80 Volts for 1 hr on 1.5% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and then visualized by UV transillumination.

2.6 DNA sequencing

2.6.1 ABI 3730 automated sequencing

Purified PCR products were used as the template in sequencing reactions using Big Dye Terminator Technology v3.1 (Applied Biosystems), which were analysed on a 3730 DNA analyser (Applied Biosystems). Each 10µl sequencing reaction consisted of 2µl ABI BigDye® terminator mix, 3.2 pmoles primer, 50ng DNA template and were made up to 10 µl with high pure H_20. The sequencing reaction was as follows: 96°C for 2 minutes, 25 × (96°C for 10 s, annealing temperature for 10 s, 60°C for 4 minutes).

Post sequencing reaction, the DNA was purified using a modified ethanol precipitation method as recommended by Applied Biosystems. DNA was added to 2.5 × volume of 100% ethanol, 0.1 volume 3 M sodium acetate (pH 5.2) and 0.1 volume of 125mM ethylenediaminetetra-acetic (EDTA). Samples were incubated on ice for 20 min, then centrifuged at 16,000g for 30 min. Supernatant was removed and the DNA pellet washed with 125µl of 70% ethanol and centrifuged at 16,000g for a further 15 min. The supernatant was removed and the DNA pellet air-dried for <20 min at room temperature while protected from light. Samples were stored at -20°C until analysis was performed on an ABI 3730 DNA analyser (SABC, Murdoch University).

2.6.2 DNA sequence assembly and analysis

Sequencing results obtained from the ABI 3730 sequencer were visually assessed using Vector NTI software (Invitrogen) and, subsequently, Geneious 4.7

(Biomatters). Splice sites were determined using the program SpliceView (http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html; verified 01.11.2011) coupled with the known bovine and human exon sequences for each gene. Contiguous sequences were created using bioinformatics software packages Vector NTI (Invitrogen), and Geneious 4.7 (Biomatters). Confirmation of sequence identify was achieved by comparison with genes and proteins from other species by GenBank NCBI BLASTn and BLASTx protocols (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7 Detection of genetic polymorphisms

SNP, insertions or deletions were identified by visual comparison of sequences between multiple alpacas. Polymorphisms were confirmed by the observation of both homozygous and heterozygous states of the mutation.

2.7.1 Predictive modeling of proteins

Several prediction models were used to analyse the potential effect of non synonymous substitution on MC1R, ASIP and TYRP1 structure and function. These included the web server versions of the scale-invariant feature transform (SIFT; Kumar et al. 2009), iMutant 2.0 (Capriotti et al. 2005), iPTree-STAB (Huang et al. 2007) and PolyPhen (Ramensky et al. 2002) algorithms. Default values were used for all programs. SIFT is based on sequence conservation and homology, using position-specific scoring matrices. It predicts the effects of amino acid substitution on both structure and function. iMutant 2.0 and iPTree- STAB use a thermodynamic method and base their predictions on protein stability using the calculated free energy change (ΔG) of mutations within the protein sequence. PolyPhen bases its predictions on empirical rules applied to the protein's sequence, phylogenetic and structural information. All methods except iPTree-STAB provide an estimate of confidence in their predictions.

3

Identification and characterisation of polymorphisms located in the *Vicugna pacos* (Alpaca) Melanocortin 1 receptor

The MC1R *gene is of interest in pigmentation studies because it is highly polymorphic and known to be responsible for colour variation in mammals. This chapter describes work in which the alpaca* MC1R *gene was analysed in a number of alpaca fibre colour phenotypes to identify potential polymorphic variants that are associated with phenotypic variation.*

3.1 Introduction

MC1R is a highly polymorphic gene consisting of a single exon spanning less than 1kb (Smith et al., 2001). This study used a candidate gene approach to identify polymorphisms with potential effects on alpaca coat colour, and *MC1R* was the first gene to be selected for investigation due to its highly polymorphic nature and critical role in mammalian pigmentation. *MC1R* has been identified as an important factor for coat colour determination in mammals. A range of loss of function and gain of function alleles have been identified in many species and these variant alleles have been definitively linked to phenotypic variation (Robbins et al., 1993, Frandberg et al., 1998, Kijas et al., 1998, Vage et al., 1999, Fontanesi et al., 2006, Theron et al., 2001, Schmutz et al., 2002). This chapter reports results obtained from sequencing of the *MC1R* gene in alpacas of various coat colour phenotypes. *MC1R* polymorphisms were then evaluated for their effect on protein structure and function and pigment type production. This was the first study to demonstrate that *MC1R* polymorphisms were associated with coat colour variation in an alpaca population (note that a portion of this work was published in Feeley & Munyard 2009).

3.2 Materials & Methods

3.2.1 Primer design

Alpaca *MC1R* primers MC1R1-F and MC1R2-R (Table 2.3) were designed to hybridise to conserved regions flanking the bovine (*Bos taurus*) and mouse (*Mus musculus*) *MC1R* sequence (GenBank accession numbers NM_174108 and NM_008559), and were used to amplify the complete alpaca *MC1R* coding region. The sequences obtained from two cloned PCR fragments were used to design alpaca-specific primers, MC1R3-F and MC1R4-R, and internal sequencing primers MC1R5-F and MC1R6-R (Table 2.3.)

3.2.2 Amplification and sequencing of alpaca *MC1R*

Initial sequence analysis, using methods detailed in Chapter 2, was performed on 9 entirely white and 15 entirely black animals. An additional 32 animals, comprising a wider range of colour phenotypes (including 5 black, 9 fawn, 4 white, 6 bay 1 chestnut, 4 black brown, 1 rosegrey and 2 silvergrey), were subsequently

analysed. Deviations from standard cycling conditions are shown in table 3.1. Sequencing reactions were performed as previously described (2.6.1) except using four primers (independently) for each product: MC1R3-F and MC1R4-R, and two alpaca-MC1R specific internal primers, MC1R5-F and MC1R6-R (Table 2.3.).

Table 3.1 *MC1R* specific PCR cycling conditions

Primer set	Extension time
MC1R1-F	1.5 min
MC1R2-R	
MC1R3-F	1.5min
MC1R4-R	

3.3 Results

3.3.1 Alpaca *MC1R* sequence

The complete coding sequence of the alpaca *MC1R* gene was generated (GenBank accession no EU135880). An NCBI BLAST search confirmed homology with *MC1R* of other species (GenBank accession number EU135880). Sequence similarity with alpaca *MC1R* was: pig and sheep 88%, goat 87%, cow 86%, human 85%, horse 84% and mouse 81%. The *MC1R* coding region contains 62% GC. Twenty-two variations were identified within the *MC1R* coding region in the 56 animals. However, only seven of the SNPs were present in more than two animals (Table 3.2 & 3.3).

3.3.2 Predicted MC1R protein

Translation of the alpaca *MC1R* sequence revealed an open reading frame of 317 amino acids. Four of the seven common SNPs caused no amino acid change (D42, N118, L206 and E311) while the remaining three resulted in amino acid substitutions (p.T28A, p.G126S and p.R301C). A 4p (c.222-225del) deletion was also identified which caused a premature stop codon p.S85X, though it was only identified in 2 animals. The alpaca MC1R protein was most similar to sheep MC1R (88%), followed by cow and cat (87%), pig (85%) and then horse and dog (84%). No correlation was observed between fibre colour alone and *MC1R* genotype in the 63 animals studied. However, when the animals were assigned to groups based on the presence or absence of eumelanin in fibre and skin, only

animals that had at least one allele with the A82/C901 combination expressed eumelanin (Tables 3.2, 3.3). Animals that did not have an A82/C901 combination (i.e. were G82/T901 or G82/Y901 genotypes) expressed only pheomelanin (Tables 3.2. & 3.3).

Table 3.2 Phenotype and *MC1R* genotypes of the initial alpaca samples examined in this study. *'E'* denotes the proposed wild type allele and *'e'* denotes the proposed recessive alleles at *melanocortin-1 receptor* (*MC1R*). Single nucleotide polymorphisms (SNPs) in bold are those that showed correlations with colour phenotype

SNP Genotype							Fibre colour	Eumelanin present	Proposed *MC1R* alleles
82	**126**	354	376	618	**901**	933			
(T28A)	**(D42D)**	(N118N)	(G126S)	(L206L)	**(R301C)**	(E311E)			
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/A	**T/T**	A/A	White	No	*ee*
G/G	**C/C**	C/T	G/G	A/A	**T/C**	A/A	White	No	*ee*
G/G	**C/C**	C/C	G/G	A/G	**T/C**	A/G	White	No	*ee*
G/A	**C/T**	C/T	G/A	A/G	**T/C**	A/G	Black	Yes	*Ee*
G/A	**C/T**	C/T	G/G	A/G	**T/C**	A/G	Black	Yes	*Ee*
G/A	**C/T**	C/T	G/A	A/G	**C/C**	A/G	Black	Yes	*Ee*
A/A	**T/T**	T/T	A/A	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	G/A	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**T/C**	A/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**C/C**	G/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	**T/T**	T/T	A/A	A/G	**T/C**	A/G	Black	Yes	*EE*

3.3.3 Statistical analysis

A fishers exact test for association was performed (In Silico; http://in-silico.net/tools/statistics/fisher_exact_test) between skin/fibre phenotype and SNP genotype. All seven SNPs were shown to have significant correlation at 2 degrees of freedom (Table 3.3). Further analysis was performed on only three SNPS (G82A, C901T and c.222-225del), as these either caused alterations to the protein sequence or were located in regions of the protein that were important in protein function.

Table 3.3 Results from fishers exact test of SNP genotype versus phenotype performed on the three SNPs that showed possible correlations between white fibre/skin, either by amino acids substitutions or by occurring in important functional domains.

SNP	Likelihood Ratio	df	Asymp. Sig (2-sided)
G82A	52.644	2	0.000
C126T	52.644	2	0.000
C901T	38.599	2	0.000

Table 3.4 Colour phenotype and *MC1R* genotypes of the additional alpaca samples examined at the three significant polymorphisms. *'E'* denotes the proposed wild type allele and *'e'* denotes the proposed recessive alleles at *melanocortin-1 receptor (MC1R*). Single nucleotide polymorphisms (SNPs) in bold are those that showed phenotypic correlations. * Eumelanin status was indeterminable. ** R301C genotype not determine due to animal being homozygous for c.222-225del.

SNP Genotype			Fibre colour	Eumelanin present	No. of Animals	Proposed *MC1R alleles*
82	126	901				
(T28A)	(D42D)	(R301C)				
A/A	T/T	**C/C**	Fawn	Yes	3	*EE*
A/A	T/T	**C/C**	Black	Yes	5	*EE*
A/A	T/T	**C/C**	Silver/grey	Yes	1	*EE*
A/A	T/T	**C/C**	Black Brown	Yes	2	*EE*
A/A	T/T	**C/C**	Silver/grey	Yes	1	*EE*
A/G	T/C	**C/T**	Bay	Yes	6	*Ee*
A/G	T/C	**C/T**	Fawn	Yes	3	*Ee*
A/G	T/C	**C/T**	Black Brown	Yes	2	*Ee*
A/G	T/C	**C/T**	White	Yes	2	*Ee*
G/G	C/C	**T/T**	Fawn	No	1	*ee*
G/G	C/C	**C/T**	Fawn	*	1	*ee*
G/G	C/C	**C/T**	Light Fawn	No	1	*ee*
G/G	C/C	**C/T**	White	No	2	*ee*
A/A	T/T	**	Chestnut	No	1	*ee*
G/G	C/C	**T/T**	Rose/grey	No	1	*ee*

3.3.4 Predicted functional effect of MC1R mutations

Three of the four prediction methods labelled the R301C polymorphism as potentially damaging to MC1R protein structure and function. Only the PolyPhen method labelled the substitution as benign.

Table 3.5 Potential effect of amino acid substitution on MC1R structure or function. *The terms 'destabilizing', 'decrease in stability' 'benign' and 'tolerate' are used by the respective methods to describe the effect of the amino acid substitution.

Amino Acid Substitution Prediction Method	Amino Acid Substitution	
	T28A	R301C
SIFT	Tolerated	Affect protein function
iMutant 2.0	Decrease in stability of structure	Decrease in stability of structure
iPTree-STAB	Destabilizing to structure	Destabilizing to structure
PolyPhen	Benign	Benign

3.4 Discussion

Analysis of results identified the G82/C126 combination as a possible marker for animals that lacked black pigment. These SNPs were correlated with the presence or absence of eumelanin in skin and fibre (Table 3.2). Genotypes A82G and C126T were in complete concordance and hence are considered to be a haplotype. All pink-skinned white animals had the G82/C126 haplotype while all black-skinned animals (regardless of fibre colour) were either heterozygotes or were homozygous for the opposite haplotype A82/T126. The animals expressing only pheomelanin are hypothesised to have the genotype *'ee'* representing the homozygous recessive genotype at *MC1R*, while the eumelanic animals are proposed to have the genotypes *'EE'* (homozygous wild type) or *'Ee',* which both allow normal eumelanin expression.

Genotypes of a larger sample group at the three non-synonymous SNPs (Table 3.3.) correlated with findings from the initial sample group. Animals that had the G82/C126 haplotype were also those animals that had an absence of black pigment in skin and fibre. The A82G polymorphism occurs in the extracellular loop of the N-terminus of MC1R (Tao, 2006, Schioth et al., 2005). Some residues in this domain have been shown to be important in normal functioning of the protein (Garcia-Borron et al., 2005). Alanine is a non-polar, hydrophobic amino acid, while threonine is a polar hydrophilic amino acid (Stoker, 2001). Substitution of alanine in place of threonine may alter the structure of the protein and inhibit its

interactions and normal functioning (Stoker, 2001). The C126T polymorphism is synonymous so it is unlikely that it has an independent phenotypic effect. However, the fact that the polymorphism displays perfect correlation with non-black pigment and that polymorphisms on either side of this SNP do not correlate perfectly with the trait, gives support to the argument that it is possibly causative of, or at least closely linked to, phenotype variation. From these results it is not clear whether these mutations are causative of a change in phenotype or merely linked to the absence of black pigment. However they may serve as a useful marker of pheomelanic animals for breeding.

C901T appeared to be a likely candidate for a role in fibre colour variation because it results in the substitution of a cysteine residue in place of arginine in the C-terminus of the protein (Schioth et al., 2005, Garcia-Borron et al., 2005). The C-terminus in GPCRs is a functionally important domain involved in interactions with the ligand–receptor complex with G-proteins (Strader et al., 1994), placement of the receptor within the membrane (Qanbar and Bouvier, 2003), and providing signals for intracellular trafficking (Schülein et al., 1998). Several studies have confirmed the link between this domain and proper receptor function (Sanchez-Mas et al., 2005b, Frandberg et al., 2001). A premature stop codon reported in the domestic dog (R306ter), which terminates the final 11 amino acids, encodes a non-functional MC1R associated with pheomelanic fibre (Newton et al., 2000). Mutagenesis studies of human MC1R have also demonstrated that the integrity of the c-terminus is critical for receptor function. Sanchez-Mas (2005b) showed that removal of the last 5 amino acids of the c-terminus was sufficient to completely abolish function, while removal of the terminal tri-peptide CSW results in a 50% reduction of available binding sites. This functional impairment was shown to be the result of reduced MC1R density on the cell surface, most likely as a result of improper trafficking of the receptor to the cell surface (Sanchez-Mas et al., 2005b).

A recent study by Ollivier et al. in 2013 reports the identification of a homologous R301C polymorphism in some breeds of ancient canids. Following this discovery R301C was subsequently identified in present day dog breeds including the Siberian Husky and Alaskan Malamute (Ollivier et al., 2013). In dogs R301C has

similarly been hypothesised as a possible causative polymorphism for lighter fibre. Interestingly, an Arginine to Serine substitution at the same amino acid position, 301, has also been reported in Mammoths (Römpler et al., 2006). In the Mammoth a tri-allelic haplotype including the R301S polymorphism is associated with a complete loss of basal activity of MC1R and a 65% reduction in activity following ligand stimulation (Römpler et al., 2006). While both these studies linked polymorphisms at position 301 to lighter fibre phenotypes, neither the effect of the mutation alone nor the functional effect of alterations at codon 301 could be established (Ollivier et al., 2013, Römpler et al., 2006).

The presence of a cysteine at residue 301 in alpacas may affect the structure of the MC1R C-terminus and prevent proper interaction of the receptor with G-proteins, resulting in a nonfunctional receptor (Frandberg et al., 2001, Sanchez-Mas et al., 2005a, Zanna et al., 2008). If these interactions are not properly enacted, downstream processes essential for the production of eumelanin are not initiated, resulting in the default melanin, pheomelanin, being produced (Hoekstra, 2006, Logan et al., 2003, Newton et al., 2000).

The c.222_225del which results in a premature stop codon at position 85 is predicted to result in a loss of MC1R function. The deletion results in a Serine at codon 85 being converted to a stop codon, where the last 232 amino acids are absent from the mature protein. Polymorphisms that result in a shortened MC1R protein are often associated with pheomelanic pigment and non-functional receptors. These include The R306t in dog (Everts et al., 2000b), the Q225X in goat (Fontanesi et al., 2009) and a premature stop coding in pigs at position 56 (Kijas et al., 2001) which are missing the last 11, 92 and 261 amino acids respectively. The truncated alpaca MC1R is predicted to exhibit a similar loss of function as it results in a receptor that is missing the last 232 amino acids. MC1R function is dependent on the strictly conserved structural domains that are common to members of the GPCR family. The S85X variant would be missing 5 TM domains, 3 extracellular loops, 2 intracellular loops as well as the C-terminus. These domains play an important role in receptor activity including receptor/ligand interaction and the C-terminus in particular is essential in MC1R function as it directs Interactions inside the cell that initiate melanogenesis (Ulloa-

Aguirre et al., 1999, Kristiansen, 2004, Tao, 2006, Hutchinson et al., 2008). A deletion of this size which is similar to that found in pigs is hypothesized to be detrimental to the protein and it is highly unlikely that the resulting protein would retain normal function.

The G376A polymorphism occurring at codon 126 is located in the central portion of the third transmembrane fragment (Garcia-Borron et al., 2005, Tao, 2006, Strader et al., 1994). This results in the substitution of a non-polar glycine by a polar uncharged serine (Stoker, 2001). It is possible that this type of substitution could alter the structure of the protein and affect its ability to function effectively (Lin and Fisher, 2007, Hoekstra et al., 2006, Tao, 2006, Jackson, 1997a, Strader et al., 1994). There are reported polymorphisms close to this position in the fox and pig *MC1R* that have been linked to phenotypic changes. Both are activating mutations resulting in dark fibre colour (Kijas et al., 1998, Vage et al., 1997). A homologous c.G376A *MC1R* mutation has been reported in the domestic duck that showed a high association with black colour phenotypes. Although the mutation showed significant association with the black phenotype in ducks, it was not determined to be the causative mutation of extended black in the domestic duck (Yu et al., 2013).

This study examined animals representing eight of over 20 recognised alpaca fibre colour phenotypes (Cecchi et al., 2004, Paul, 2006) and both coat types (Suri and Huacaya). Determining correlations between polymorphisms and phenotype in alpacas is difficult because of the complex and multi-staged interactions of pigment-related genes. There are currently 378 known genes involved in the pigmentation process with the potential for colour to be affected at many stages (Bennett and Lamoreux, 2003, Montoliu et al., 2014). This is evident in our results as some animals with an identical *MC1R* genotype displayed different phenotypes (Tables 3.2, 3.3). It is probable that other genes are acting to modify coat colour in these animals. The epistatic relationship between *MC1R* and the *Agouti* gene makes it especially difficult to make predictions on the effect of such *MC1R* polymorphisms without knowledge of the *Agouti* genotype (Furumura et al., 1996, Lin and Fisher, 2007). Agouti is effective only in the presence of a fully functioning MC1R and can cause a range of colours and

patterns including completely eumelanic and completely pheomelanic (Girardot et al., 2006, Kerns et al., 2003, Voisey et al., 2001, Rieder et al., 2001). This may explain the presence of pheomelanic animals in this study that had an apparently similar phenotype but different genotype. When both the Agouti signaling protein and a fully functioning MC1R are present, Agouti signaling protein can act to switch production from pheomelanin to eumelanin (Kerns et al., 2003, Hart, 2001, Paul, 1999).

Colour dilution genes may also account for the pigment variation displayed between animals with identical *MC1R* genotypes. Pigmentation is a multi-stage process with the potential for pigment to be affected at any stage (Busca and Ballotti, 2000, Hoekstra, 2006, Lin and Fisher, 2007). Even in the presence of a fully functioning MC1R receptor, if the genes responsible for packaging and transport of melanosomes are not functional or efficient it may result in an overall dilution of the manufactured pigment simply due to the inability of the melanosome to exit the cell and be transported into the surrounding keratinocytes (Potterf et al., 1998, Guibert et al., 2004, Le Pape et al., 2008). Mutations in the promoter sequence or mutations in transcriptional elements of *MC1R* may also be responsible for the differences seen in phenotype between animals with identical *MC1R* genotypes. Polymorphisms within the promoter regions of *MC1R* with effects related to pigmentation have been reported in both humans (Motokawa et al., 2008) and sheep (Furumura et al., 2001, Guibert et al., 2004).

Mutations that affect the functioning of promoters and certain transcription factors have been shown to result in an increase in melanin synthesis (Murisier et al., 2006). GPCR promoters are characteristically GC rich and lack a TATA box (Moro et al., 1999). Characterisation of the *MC1R* promoter was not included in this study and it remains a significant target area for the further investigation of fibre colour variation in alpacas. An important factor that must be considered in alpaca coat colour studies is the level of genetic diversity of the animals sampled, which has been illustrated by Powell et al. (2008) in a study of *MC1R* in alpacas in the USA. There were seven SNPs common to both that work and this study; however, four SNPs that appeared in their sample population were not found

here. It is possible that some phenotypically relevant polymorphisms existing in the Australian alpaca population were not identified in the current study, or that reduced genetic diversity has removed some from the population. Interestingly Powell (2008) reported similar results to this study without finding associations with colour phenotypes. This is most likely a result of the differing approach to phenotype assignment and how it was used to interpret genotypic data.

3.5 Conclusion

This series of experiments has provided new information on the possible effects of *MC1R* alleles in alpaca fibre pigmentation. The results have characterised a haplotype that appears to be a significant marker for the absence of black pigment and has significant potential for use as a marker in breeding stock selection. Investigation and characterisation of the *MC1R* promoter may also yield information useful in analysing the differences in *MC1R* expression in animals with identical genotypes that display varying degrees of pigmentation. To further understand the influence of these *MC1R* polymorphisms in pigmentation, a functional investigation of the receptor activity was undertaken; these results are described in chapter 4.

4

Functional characterisation of polymorphic variations in the alpaca *MC1R* gene

In Chapter 3, it was shown that there was an association between alpaca coat colour phenotypes and MC1R *polymorphisms. Based on what is known about the activity of G protein coupled receptors and the regions of the protein in which these substitutions occurred it was suggested that these effects may be due to the reduced ability of polymorphic variant receptors to elicit adequate intracellular responses, such as cyclic AMP production. This chapter investigates the functional activity of these polymorphic variants.*

4.1 Introduction

The MC1R protein is part of a large family of membrane bound peptides known as G protein coupled receptors (GPCRs) (Gether, 2000, Kobilka, 2007, Rouzaud and Hearing, 2005). These proteins share a common membrane architecture consisting of an extracellular N-terminus, seven transmembrane-spanning regions joined by three extracellular and three intracellular loops, with an extracellular C-terminus (Gether, 2000, Kobilka, 2007). While sharing significant structural similarity within the GPCR family, each individual receptor is activated by highly specific ligand molecules (Ulloa-Aguirre et al., 1999, Bockaert et al., 2002, Kristiansen, 2004).

Intracellular cyclic AMP (cAMP) production is commonly used as a measurement of GPCR activity (Busca and Ballotti, 2000, Newton et al., 2005, Thomsen et al., 2005). Binding of a ligand to the GPCR induces a conformational change in the receptor that promotes G-protein coupling, and the subsequent activation of the adenyl cyclase pathway (Strader et al., 1994, Gether, 2000, Schiaffino, 2010). In the pigmentation pathway, low levels of cAMP result in basal pheomelanogenesis, while high levels of intracellular cAMP stimulate melanin synthesis and favour the production of eumelanic pigment (Ito and Wakamatsu, 2003). Reduced functionality of MC1R, which is the proposed outcome of the alpaca MC1R polymorphisms reported in chapter 3, would result in a melanocyte capable of producing only pheomelanin.

This chapter describes experiments used to measure the ability of mutant MC1R receptors to stimulate cAMP production and discusses the results. Experiments were conducted to investigate the functional consequences of the T28A and R301C MC1R alleles on receptor activity. These polymorphisms, identified in the alpaca MC1R, showed an association with non-eumelanic pigment phenotypes, although the mechanisms responsible for this effect could not be determined with the information generated by sequencing alone. The S85X polymorphism also described in chapter 3 was not analysed as it was discovered in the late stages of the project after the functional analysis of MC1R had been completed.

4.2 Materials and Methods

4.2.1 Site directed Mutagenesis and MC1R construct design

The wild type human MC1R, cloned into expression vector pcDNA3.1+, was purchased from Missouri S&T cDNA Resource Center (Genbank accession NM_002386). This human MC1R construct was used as the template in PCR-based site-directed mutagenesis reactions to create three MC1R constructs that replicate two previously described MC1R mutations in alpaca *MC1R,* T28A and R301C (Feeley & Munyard 2009). PCR primers were designed using human *MC1R* sequence to incorporate a Thr28, or Cys301 substitution. As the human wild type MC1R already contained an alanine at residue 28, this residue was mutated to a threonine to assess the affect of amino acid substitutions at this residue. A third construct was created that contained both substitutions Thr28/Cys301, to mimic the putative '*e'* recessive *MC1R* allele in alpacas.

Mutagenesis was performed in 50μl reactions containing reaction buffer (1.2 M Tris.HCl, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 1% Triton X-100, 0.01% BSA, pH8.0), 1.5mM $MgCl_2$, 0.4mM of each primer and 1U KOD Hotstart polymerase. PCR amplification was performed using GeneAmp PCR System 9700 (Applied Biosystems®) and cycle conditions were initial denaturation at 95°C for 2 min, followed by 20 cycles, each consisting of 95°C for 30s and 70°C for 3.5 min; with a final extension at 70°C for 3 min. Oligonucleotides used for mutagenesis are shown in table 4.1. Successful mutation incorporation was confirmed by DNA sequence analysis using Big Dye terminator technology (Applied Biosystems, Scoresby, Vic., Australia), vector specific sequencing primers (Table 4.1.) and analysis on a 3730 DNA analyzer (Applied Biosystems).

Table 4.1 Oligonucleotide primer sequences for *MC1R* site directed mutagenesis reactions

Construct	Mutation	Primer	Sequence
MC1R28	A28T	T28-F	5' GCTGGCTACCAACCAGACAGGAG 3'
		T28-R	5' CTCCTGTCTGGTTGGTAGCCAGC 3'
MC1R301	H301C	C301-F	5' CATCTACGCCTTCTGCAGCCAGGAGC 3'
		C301-R	5' GCTCCTGGCTGCAGAAGGCGTAGATGAGGG 3'
Sequencing Primers		T7-F	5' TAATACGACTCACTATAGGG 3'
		BGH-R	5' TAGAAGGCACAGTCGAGG 3'

4.2.2 Cell culture and transfection

HEK293 cells were maintained at 37°C, 5% CO_2 in complete medium (DMEM containing 0.3mg/ml glutamine, 100IU/ml penicillin and 100μg/ml streptomycin; GIBCO BRL, Carlsbad, CA) supplemented with 10% foetal calf serum (GIBCO). Transient transfections were performed 24 hours after seeding with GeneJuice (Merck, Kilsyth, Australia) according to the manufacturer's instructions.

4.2.3 Measurement of cAMP production

HEK293 cells were seeded in 6-well plates at a density of 200,000 cells per well. At 24 hours after transfection, cells were harvested in complete medium, added to a poly-$_L$-lysine-coated 48-well plate and incubated at 37°C, 5% CO_2. At 48h after transfection, the cAMP production from cells stimulated with 1μM α-MSH (treated sample) or with solvent alone (untreated sample) was measured using the cAMP AlphaScreen assay kit (PerkinElmer) according to the manufacturer's instructions. The AlphaScreen signal (counts per second) was measured in 384-well microplates on an Envision Multilabel Plate Reader (PerkinElmer) and was used to calculate the concentration of cAMP using a standard curve.

4.2.4 Statistical analysis

To identify significant responses to ligand stimulation, data were normalized relative to the respective control and assessed for statistically significant

differences from 100% using a two-way ANOVA with Bonferroni's post-test. Statistical analysis of results was performed using Prism software (GraphPad Software, Inc., San Diego, CA).

4.3 Results

4.3.1 Effect of MC1R variants on the ability of α-MSH to elevate intracellular cAMP levels

Receptor-mediated cAMP production was measured to assess the ability of different variants of MC1R to activate the cAMP pathway. The results demonstrated that ligand-induced cAMP accumulation was significantly decreased with all three MC1R mutants. The MC1R28 mutant showed a 44% decrease in cAMP accumulation when compared to the wild-type receptor, while the MC1R301 and MC1R28/301 mutants showed an 88% and 93% reduction in signalling, respectively (Figure 4.1). The study was not able to demonstrate a significant difference between basal and ligand stimulated cAMP accumulation in the wild type MC1R but a significant difference was observed in basal signalling and ligand stimulated cAMP production between MC1R-301 and the wild type receptor (Figure 4.2)

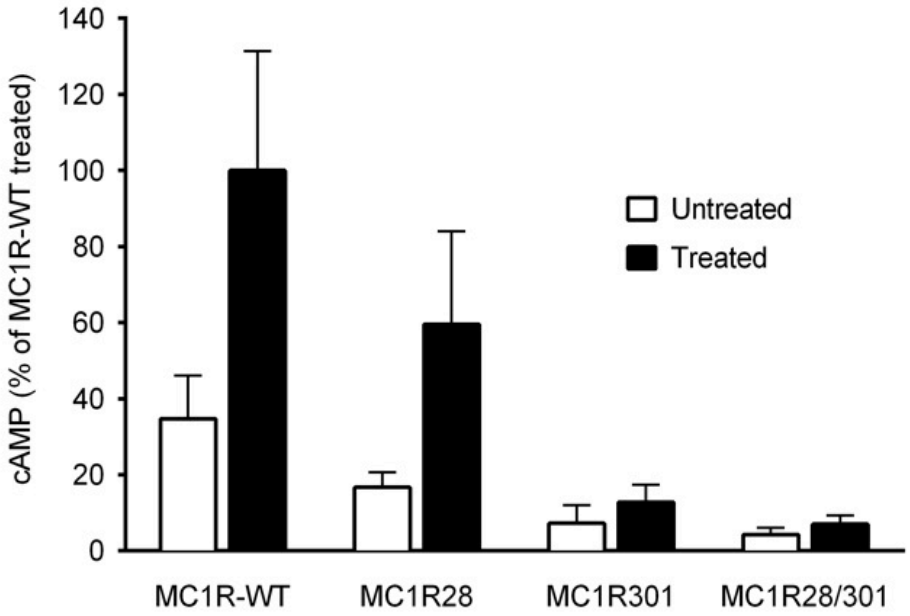


Figure 4.1. Effect of MC1R substitutions on intracellular cAMP production. Cells were treated with α-MSH or solvent alone for 15 minutes prior to cell lysis and detection using the AlphaScreen cAMP assay system. Results are expressed as a percentage of maximum wild-type level upon ligand stimulation.

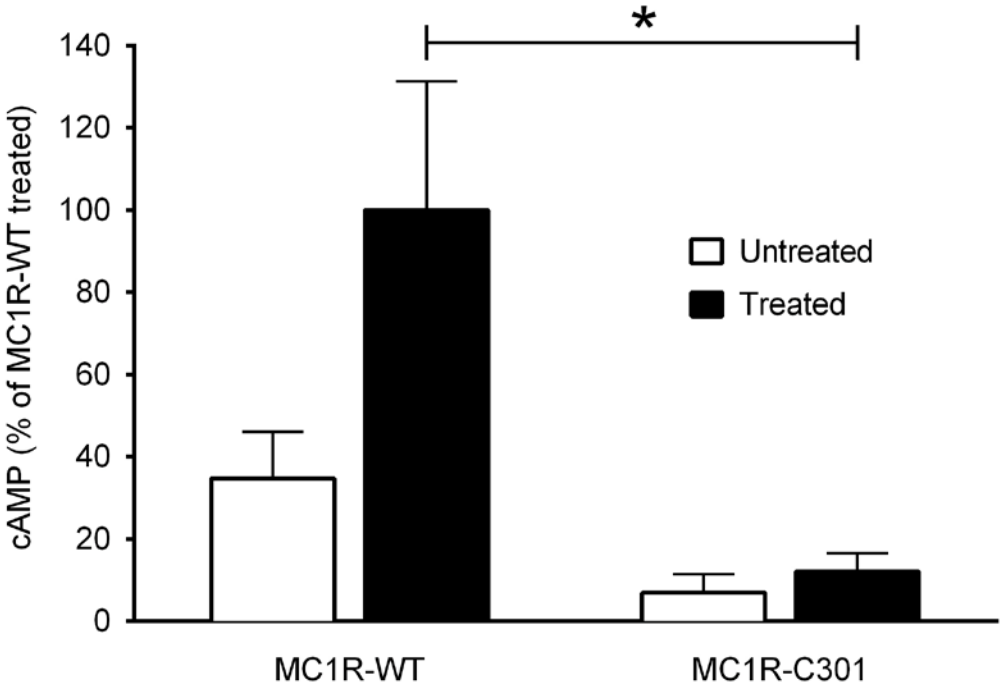


Figure 4.2. Significant effect of MC1R substitutions on intracellular cAMP production compared to wild type MC1R receptor. Results are expressed as a percentage of maximum wild-type level upon ligand stimulation. Data values are the mean of three independent experiments (* $p<0.05$).

4.4 Discussion

Mutation of the His301 residue in the wild-type human MC1R protein to cysteine resulted in a significant impairment of receptor activity, with an 88% reduction in cAMP accumulation when compared to the wild type receptor (Figure 4.1). The dramatic reduction in signalling is not surprising given results discussed in chapter 3 that demonstrated an association between the corresponding alpaca MC1R polymorphism, R301C, and pheomelanic fibre colour phenotypes (Feeley and Munyard, 2009). The Arginine residue at codon 301 is likely to be important in MC1R function as when this residue is substituted in other species including both dog and mammoth it is similarly associated with light fibre (Ollivier et al., 2013, Römpler et al., 2006).

The homologous R301C in dogs has been reported to correlate with lighter fibre phenotypes (Ollivier et al., 2013). In the mammoth an arginine to serine substitution at reside 301 has been examined as a possible cause for light fibre phenotypes. Functional analysis of an MC1R haplotype that included the polymorphism at residue 301 was able to almost completely abolish basal signalling of the receptor and lead to a 65% reduction in receptor signalling upon ligand stimulation (Römpler et al., 2006). This reduction of MC1R activity following ligand stimulation was shown to be associated with lighter fibre colour however these effects were attributed to the haplotype and not to one single polymorphism. This combined evidence suggests that the 88% reduction in cAMP production seen in this study would be comparable to an inactive receptor and lead to intracellular conditions that support the exclusive production of pheomelanic pigment.

R301C occurs in the highly conserved C-terminal domain, which is known to have an important role in receptor activity (Garcia-Borron et al., 2005, Sánchez-Más et al., 2005). The exact mechanism by which the C301 substitution inhibits receptor

function has not been determined. One possible explanation for the reduction in cAMP accumulation seen may be a reduction in the density of receptors on the surface of melanocytes, due to an inability of the receptor to be correctly transported to the cell membrane. A lower number of receptors in the cell membrane, and therefore fewer receptors available for interaction with the ligand, would likely result in an overall reduction in intracellular cAMP production (Sanchez-Mas et al., 2005b). The human red hair variant alleles R151C and R160W have been shown to result in an 85% reduction in cell surface binding compared to the wild type receptor which is reported as the cause of low cAMP production in these variants (Beaumont et al., 2005). A second possible cause of reduced signalling could be through the substitution interrupting or inhibiting proper G protein coupling, as is seen in the non functional D294H allele in humans. This MC1R human variant shows high cell surface expression but poor G-protein coupling and results in a significant reduction in receptor signalling (Beaumont et al., 2005). Reduced or improper coupling would prevent the activation of the adenyl cyclase pathway, leading to a decrease in cAMP accumulation, which would generate intracellular conditions that support pheomelanin synthesis (Ulloa-Aguirre et al., 1999, Schoneberg et al., 2004, Newton et al., 2005, Gether, 2000). It is not possible from these results to establish the exact mechanism by which the alpaca R301C polymorphism is affecting MC1R function.

While there is certainly a marked reduction in cell signalling in each of the MC1R mutants there was no significant difference found between basal signalling and ligand stimulated cAMP production in the wild type receptor (Table 4.2). As a result it is not possible to definitively establish that the marked reduction in cAMP production seen in each of the MC1R mutants is a result of the polymorphism and not an effect of the experimental process of introducing the mutated residue. Before these polymorphisms can be established as non functional variants it would be necessary to include a receptor binding assay to provide evidence that the cells were stably transfected with MC1R and that the receptor was expressed on the cell surface. Given that the difference between basal and α-MSH-stimulated production of cAMP cannot be distinguished, then all that can be

confirmed is that the polymorphisms, or the process of mutagenesis and transfection, affected constitutive production of cAMP.

The wild-type receptor used in this study was a human MC1R protein. Residues 28 and 301 were mutated, which corresponded to Thr28Ala and R301C in the alpaca protein. Use of the human protein was not ideal, however the alpaca receptor was not available, and there is significant similarity with 85% conservation of amino acid residues between the human and alpaca MC1R. This similarity indicates that the human receptor would serve as a suitable substitute for predicting the effect of the substitution of these residues on receptor activity. However, the wild type human receptor used in this study already contained an alanine residue at codon 28, which was associated with a loss of function phenotype in the alpaca MC1R. As a consequence, the T28 variant was instead analysed to measure the effect of changing this residue on the activity of the receptor.

We found that the T28 variant showed a 44% reduction in cAMP production, though this was not comparable to the 88% reduction observed following the substitution of cysteine at codon 301 (Figure 4.1). These results indicated that this residue might have an important role in maintaining receptor function, as there was a significant reduction in cAMP production as a result of the substitution; however it is not clear if disruption of this residue alone would be sufficient to render the receptor completely non-functional. Additionally, it is difficult to make accurate assumptions as to the effect of the T28A polymorphism on MC1R function as the wild type human MC1R already contained the predicted loss of function alanine at that residue. However the finding that a substitution at this residue in the human receptor results in a reduction in cAMP accumulation indicates that this residue may have an import function for receptor activity and should not be discounted.

The contribution of each polymorphism to receptor activity was analysed in individual constructs, (1) and (2). We also measured the combination of both substitutions in a single construct. The compound mutation (3) resulted in 93% reduction in cAMP accumulation when compared to the wild type receptor. This

result provides evidence that the Cys301 substitution is responsible for the severely reduced receptor activity, despite the fact that both the A28 and C301 substitutions showed correlation with loss of function phenotypes in the previous study (Feeley and Munyard, 2009). It is more likely that the A28 substitution is contributing a cumulative effect to the reduced receptor activity and alone is not sufficient to cause a complete loss of function.

Loss of function mutations in MC1R that are associated with phenotype variations have often been reported in mammals (Everts et al., 2000a, Fontanesi et al., 2010a, Grimes et al., 2001, Markland et al., 1996, Schioth et al., 1999). The confirmation that the A28/C301 polymorphisms are associated with loss of receptor activity has important implications for the alpaca breeding industry. These mutations are valuable because they can be utilised as a marker to identify animals that are either carriers or homozygous for this type of pheomelanic genotype. This information will have an important role in improving prediction of outcomes when breeding for selected phenotypes.

4.5 Conclusion

This study provides further evidence that there are non functional alleles in the alpaca *MC1R*. The results from this study show that the Cys301 polymorphism is the predominant contributor to reduced cAMP activity of MC1R, most likely due to a reduction in receptor availability or via poor G protein coupling. This provides further evidence to support the claim that the A28/C301 MC1R allele, identified in the Australian alpaca population, is a loss of function allele and is responsible for non-eumelanic phenotypes.

5

Investigation of the role of *Tyrp1* in alpaca fibre pigmentation

The Tyrp1 *gene has been identified as the locus responsible for brown phenotypes in a number of species including mice, cattle, sheep and cats. It is known as the "*brown*" locus and results in a characteristic chocolate brown phenotype. Polymorphisms that disrupt the enzymatic activity of Tyrp1 lead to production of brown eumelanin rather than black eumelanin by preventing the oxidation of DHICA, which is necessary for the synthesis of the final eumelanin polymer. This chapter discusses the investigation of variation in the alpaca* Tyrp1 *gene to determine if it is responsible for the brown phenotypes that exist in the Australian alpaca population.*

5.1 Introduction

The role of *Tyrp1* in alpaca pigmentation is currently unknown. The presence of brown eumelanic pigment has been proposed in alpacas because of the common breeding outcome in which two black parents produce a brown cria. The *Tyrp1* gene is a strong candidate gene for some or all of the brown fibre colour phenotypes that are observed in alpacas, and is a possible candidate for the variation in intensity of colour observed among eumelanic animals.

The lack of distinct boundaries between the 22 recognised alpaca colour groups and the subjectivity of phenotype assignment makes colour studies more difficult. Consequently *Tyrp1* sequence analysis was conducted in conjunction with the chemical analysis of melanin content in alpaca coat fibres. The melanin analysis assay, using the method described by Ozeki & colleagues (1996), measures follicular melanin following solubilisation in Soluene-350. This method enables quantification of eumelanin/pheomelanin ratios and an estimate of total melanin content. The chemical properties of melanin in animal fibre have been the focus of several studies in other species including mouse (Ozeki et al., 1995), sheep, goat (Sponenberg et al., 1998) and llama (Cecchi et al., 2007, Sponenberg et al., 1998).

5.2 Materials & Methods

5.2.1 Animal selection

Initial sequence analysis, using methods detailed in Chapter 2, was performed on 4 black, 5 white and 19 putative eumelanic brown animals. *Tyrp1* polymorphisms in other species display a characteristic phenotype often termed warm brown or chocolate (Lyons et al., 2004). Following this premise, the brown sample group was selected to contain animals that were most likely to be eumelanic brown, either through pedigree or visual inspection of coat colour. After the initial sequence analysis, a further 18 animals (8 black, 6 white and 4 brown) were genotyped for all exon 4 mutations. Fibre sample were collected from 36 of these animals. While it is known that the activity of Tyrp1 is confined to eumelanic pathway, and hence pheomelanic animals will not express *Tyrp1* alleles, 10 white

animals were included in this study. Their selection was based on the colour of the parents rather than the fibre colour of the offspring so that we might identify *Tyrp1* alleles existing in these animals even though they would be masked in white fibre animals.

5.2.2 Primer walking (Exon 4)

No sequence data were available for an approximately 2.5 kb region of the alpaca *Tyrp1* gene which, based on surrounding sequences and known *Tyrp1* intron/exon boundaries of other species, was hypothesised to contain exon 4. In order to obtain the missing sequence, primers were designed to amplify a 2.5kb section of the *Tyrp1* gene, and then sequence the ends of this fragment. The sequence obtained was then used to design a series of internal primers in approximately 500bp intervals to "primer-walk" through the unknown sequence.

Table 5.1 Internal primers used to sequence a 2.5kb region spanning exon 4 in alpaca *Tyrp1*

Tyrp1 Exon	Primer set		Tm °C
Ex4F2	5' CCTCTGTAGTCTGTAGTCAT 3'	Seq	60°C
Ex4R2	5' AAGAGTTTGGGATTGGCAGA 3'	Seq	60°C
Ex4R3	5' TCCAGTGATCTGAGTGCCAC 3'	Seq	60°C
Ex4F3	5' AACGTTTATTCTGCGTATGTTTTT 3'	Seq	60°C
Ex4F4	5' GCTTCACACCAAAACCCACT 3'	Seq	60°C

5.2.3 Melanin assay

The spectrophotometric analysis of melanin content was performed according to the method of Ozeki *et al.* (1996), on 36 fibre samples from the animals genotyped in this study. Fibre samples were unavailable from the remaining 10 animals. Samples were assayed in triplicate and the results averaged.

5.3 Results

5.3.1 The alpaca *Tyrp1* gene

The complete coding sequence of the alpaca *Tyrp1* gene was generated using the sequencing protocols detailed in chapter 2 (Genbank accession no. JN122622). A BLAST search confirmed similarity with *Tyrp1* of other species, with the highest sequence similarity being to pig (90%), followed by cow, sheep and goat (88%) and human (87%). Alpaca *Tyrp1* consists of seven coding exons, which was confirmed by examination of exon splice sites and comparison to mouse and human *Tyrp1*. The location and size of each exon and the intronic regions is shown in Figure 5.1. Exons 2-8 contain 57, 50, 46, 50, 46, 39 and 45% GC (respectively) and the whole coding region is 49% GC. *Tyrp1* has a short non coding exon, termed exon 1 which is located variable distances in different species upstream of the start codon in exon 2. Alpaca exon 1 was not examined in this study.

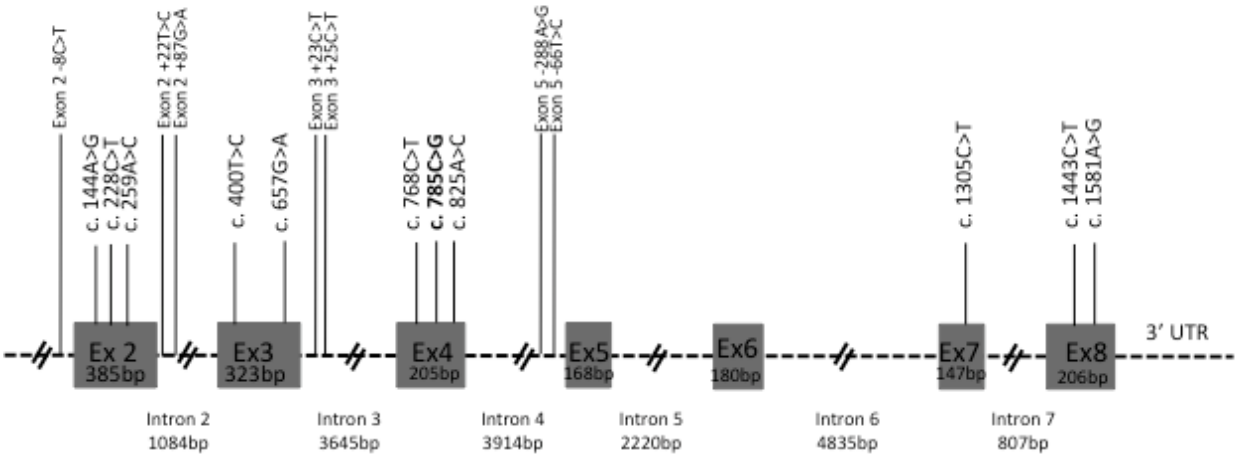


Fig. 5.1. The alpaca *Tyrp1* gene. Coding exons are shown as solid boxes. Intronic regions are shown as dashed lines (not to scale). Exon 1 in *Tyrp1* is an untranslated exon which is located upstream of the start codon, which is located in what is most often referred to as exon 2 (Jackson et al., 1991, Sturm et al., 1995, del Marmol and Beermann, 1996). Polymorphisms are shown in the regions where they occur; non synonymous polymorphisms are shown in bold. Indicated intron size was determined from the Ensembl genome assembly and new sequence data generated in this study.

5.3.2 Alpaca predicted alpaca Tyrp1 protein

In silico translation of alpaca *Tyrp1* revealed a 537 amino acid predicted protein (Fig. 5.2) that shares greatest similarity with Tyrp1 of pig and cow (91%), and sheep and goat (90%). The consensus prediction for the signal peptide was from residues 1 to 24, with the predicted cleavage site between residues 24(A) and 25(Q). Analysis of the predicted amino acid sequence identified two copper binding domains, a transmembrane domain and an epidermal growth factor (EGF)-like domain. The proposed locations of these domains are shown in Fig 5.2.

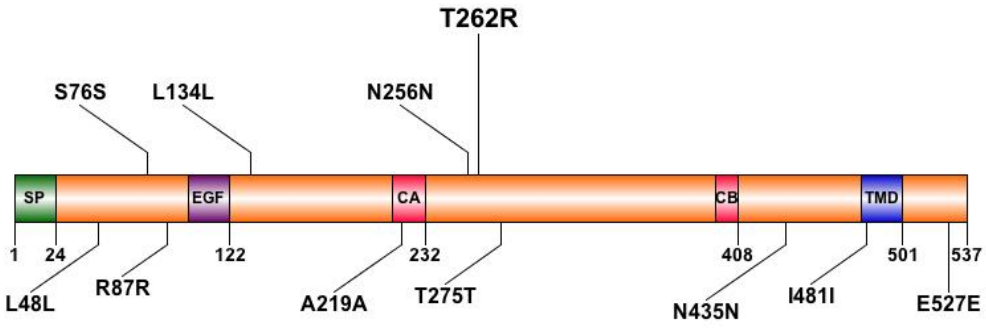


Fig. 5.2. Schematic illustration of the predicted alpaca Tyrp1 protein. Areas representing the signal peptide (SP), epidermal growth factor (EGF)-like domain, copper binding domains A and B (A, B), and transmembrane domains (TMD) are indicated. The 11 SNPs identified within the coding regions are shown in the regions in which they occur. The only non-synonymous polymorphism identified, T262R, is highlighted.

5.3.3 Polymorphic variation in alpaca *Tyrp1*

Eleven novel polymorphisms were identified in alpaca *Tyrp1* exons (Table 5.2,

Figure 5.1), including only one non-synonymous SNP in exon 4; c.C785G, which is predicted to cause a threonine–to-arginine substitution at codon 262 (Figure 5.1). An additional eight SNPs were identified within the intronic regions sequenced. No correlation was observed between any *Tyrp1* polymorphism and the fibre colours examined (Table 5.2. & 5.3.).

Table 5.2 Polymorphisms identified in the alpaca *Tyrp1* gene. The only non-synonymous polymorphism is shown in bold.

Polymorphism	Location	Amino acid affect	p values
Exon 2 -8C>T	5' UTR	N/A	0.491
c. 144A>G	Exon 2	L48L	0.264
c. 228C>T	Exon 2	S76S	0.671
c. 259A>C	Exon 2	R87R	0.403
Exon 2 +22T>C	Intron 2	N/A	0.999
Exon 2 +87G>A	Intron 2	N/A	0.239
c. 400T>C	Exon 3	L134L	0.217
c. 657G>A	Exon 3	A219A	0.051
Exon 3 +23C>T	Intron 3	N/A	0.724
Exon 3 +25C>T	Intron 3	N/A	0.841
c. 768C>T	Exon 4	N256N	0.060
c. 785C>G	**Exon 4**	**T262R**	**0.909**
c. 825A>C	Exon 4	T275T	0.200
Exon 5 -288A>G	Intron 4	N/A	0.513
Exon 5 -66T>C	Intron 4	N/A	0.574
c. 1305C>T	Exon 7	N435N	0.193
c. 1443C>T	Exon 8	I481I	0.110
c. 1581A>G	Exon 8	E527E	0.180
Exon 8 +8C>T	3' UTR	N/A	0.085

Table 5.3 *Tyrp1* genotypes of all polymorphisms identified in the alpaca *Tyrp1* coding sequence. Fibre colour is White (W), Black (Blk), Dark Brown (DB), Brown (B) or Red Brown (RB).

EXON 2			EXON 3		EXON 4			EXON 7	EXON 8		Fibre Colour	Number of Animals
144	228	259	400	657	768	**785**	825	1305	1443	1581		
L48L	S76S	R87R	L134L	A219A	N256N	**T262R**	T275T	N435N	I481I	E527E		
G	T	A	T	A	C	**G**	A	T	T	A	W	1
AG	CT	A	T	GA	CT	**CG**	AC	CT	CT	A	W	1
AG	CT	A	T	G	CT	**CG**	AC	CT	C	A	DB	1
G	CT	AC	T	G	CT	**CG**	AC	CT	C	A	B	1
AG	CT	A	T	G	CT	**C**	AC	CT	C	A	RB DB	2 1
AG	CT	A	T	GA	CT	**C**	AC	CT	CT	A	W DB	2 1
AG	CT	A	T	G	T	**C**	C	C	C	A	DB	1
AG	CT	A	T	GA	CT	**C**	AC	CT	CT	A	B	1
AG	C	AC	T	GA	CT	**C**	AC	C	CT	A	Blk	1
AG	C	AC	T	G	T	**C**	C	C	C	A	DB	1
AG	C	AC	TC	G	CT	**C**	C	CT	C	A	DB	1
A	C	A	T	G	T	**C**	C	C	C	A	B	2
A	C	A	TC	GA	CT	**C**	C	CT	C	A	B	1
G	C	C	TC	G	CT	**C**	C	CT	C	A	Blk	1
G	C	C	TC	G	CT	**C**	C	C	C	AG	Blk	1
G	C	C	T	G	T	**C**	C	C	CT	A	DB	1
G	CT	AC	TC	GA	C	**C**	AC	T	C	A	Blk	1
G	CT	AC	T	GA	CT	**C**	AC	CT	CT	A	W B DB	1 2 1
G	C	AC	T	G	CT	**C**	AC	CT	C	A	DB	1
G	CT	AC	T	G	CT	**C**	AC	CT	C	A	DB	1

Table 5.4 *Tyrp1* exon 4 genotypes for additional animals analysed in this study. Fibre colour is White (W), Black (Blk), Dark Brown (DB) or Brown (B).

Exon 4			Fibre Colour	No. of animals
768	**785**	825		
N256N	**T262R**	T275T		
CT	**CG**	AC	W B	1 1
CT	**C**	AC	W B	1 2
CT	**C**	C	W	2
T	**C**	C	W Blk DB	1 6 1
C	**C**	AC	W	1
C	**C**	A	Blk	1
C	**CG**	AC	Blk	1

5.3.4 Predicted functional effect of Tyrp1 mutations

All the amino acid substitution prediction methods employed predicted that the T262R substitution was potentially damaging or destabilizing to Tyrp1 structure or function (Table 5.5).

Table 5.5 Predicted effect of the amino acid substitution, T262R, on Tyrp1 structure and function. * 'Destabilizing' and 'probably damaging' are terms used by prediction programs to describe the effect of substitutions on the protein.

Amino acid substitution prediction method	Amino acid substitution T262R
SIFT	Likely to affect function
iMutant 2.0	Decrease in stability of structure
iPTree-STAB	Destabilizing* to structure
PolyPhen	Probably damaging* to structure and function (high confidence)

5.3.5 Melanin assay

The amount and type of melanin present in the fibre samples used in this study was measured (Figure 5.3). All white samples fell below the 0.15 A650/500 ratio predicted for predominantly pheomelanic samples, and all black fibre samples were above the 0.25 A650/500 ratio predicted for predominantly eumelanic samples. The A650/500 for brown samples ranged between 0.16 and 0.45.

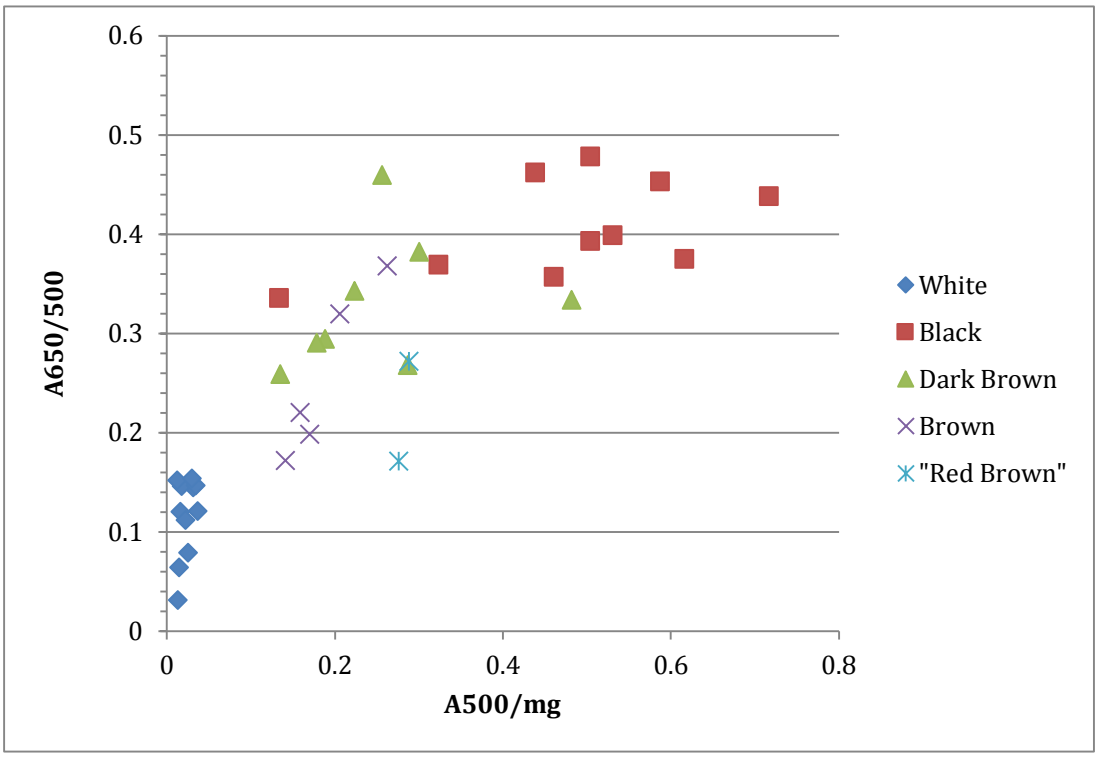


Figure 5.3. Total melanin compared with A650/500 ratio, a measure of the relative proportion of melanins present in alpaca fibre. Ratios are indicative of a gradient in melanin type with >0.25 indicating predominantly eumelanic pigment, <0.15 indicating predominantly pheomelanic pigment, and ratios >0.15 but <0.25 indicative of a mixture of both pigment types. Each data point represents the average of three replicates for each sample.

5.4 Discussion

The only non-synonymous SNP found in *Tyrp1*, T262R, did not have any significant association with brown fibre colour phenotype in alpacas (p=0.909). While amino acid substitutions are a common cause of phenotypic variation, it does not appear that T262R is responsible for the brown phenotype in alpacas.

Protein structure has been demonstrated to have important consequences for function. Disruptions that interfere with protein folding or inhibit essential protein-protein interactions often result in a loss of protein function (Wang and Moult, 2001, Collins et al., 1997). Automated prediction methods can be a useful tool for predicting potentially damaging amino acid substitutions (Ng and Henikoff, 2006, Chasman and Adams, 2001). In order to assess the effect of the

threonine to arginine substitution on the alpaca Tyrp1 protein, four different prediction programs were employed. The consensus prediction was that the arginine substitution would likely be destabilizing or damaging to the protein structure, and would therefore likely affect function (Table 5.4). However this prediction does not conform to the current results because no correlation was found between the T262R polymorphism and brown fibre colour. An alternative explanation is that although the T262R substitution may be compromising Tyrp1 activity, the effect is unrelated to pigment and as a result no correlations were observed. It may also be possible that the 'brown' animals analysed in this study were a mix of eumelanic brown animals and pheomelanic animals that appear brown and because they were analysed as one phenotype any correlation may have been masked. However the latter scenario is unlikely as fibre analysis of these animals did not identify any eumelanic brown animals. While Tyrp1 has known functions as a DHICA oxidase and also in the stabilisation of tyrosinase (Kobayashi et al., 1998, Kobayashi et al., 1994) it is possible that the protein has further unknown functions.

The Tyrp1 protein has several structural features which are important for function, including a cysteine-rich epidermal growth factor (EGF)-like domain that is involved in protein-protein interactions, and two copper binding sites which have an important role in the catalytic reactions of melanin synthesis (Furumura et al., 1998, Kobayashi et al., 1998). Base substitutions that occur within these important functional domains are more likely to have a damaging effect on the protein. The p. T262R variant does not occur in a region that has been identified as having functional significance for the protein (Figure 5.2). While prediction tools can be informative, it is important to consider that they are predictive modeling tools as opposed to confirmed experimental data and should be interpreted as such. It seems highly unlikely that this polymorphism is responsible for fibre colour variation in alpacas, as demonstrated by both the lack of significant association of T262R with fibre colour and by the finding that the polymorphism does not occur in an important functional domain.

Another scenario that may explain the lack of association observed between alpaca *Tyrp1* mutations and the brown fibre colour phenotype is the possibility

that there is more than one mutation causing genetic brown. Multiple *Tyrp1* mutations have been reported in dogs, which exhibit three *Tyrp1* brown mutations (Schmutz et al., 2002) and in cats, in which three *Tyrp1* mutations are linked to brown phenotypes (Lyons et al., 2004, Schmidt-Küntzel et al., 2005). It is also possible that the brown phenotype observed in alpacas is being controlled by the actions of another gene entirely. In genetic terms the brown phenotype, also known as chocolate, liver or cinnamon, refers specifically to brown eumelanin, which occurs when there is a higher concentration of DHICA derived melanins (Kobayashi et al., 1994, Jiménez-Cervantes et al., 1994). The *Tyrp1* brown phenotype is seen in chocolate Labrador Retrievers (Schmutz et al., 2002) and also in mice (Zdarsky et al., 1990). The lack of a common nomenclature for phenotype descriptions and the comparison of fibre colour between different species has led to the 'brown' phenotype being incorrectly used to describe animals which are in fact intensely pigmented pheomelanic animals. It may be the case in alpacas that the 'brown' phenotype breeders describe is actually pheomelanic, and therefore not likely to be a result of the *Tyrp1* gene whose actions are confined to the eumelanic pathway (Hearing and Tsukamoto, 1991, del Marmol et al., 1993, Kobayashi et al., 1995).

In addition to *Tyrp1* sequence analysis, analysis of the chemical composition of the fibre was performed. It has been hypothesized that most brown alpacas are agouti variants (Munyard, 2011) in which case their fibre would be either pheomelanic or mixed pheomelanin and eumelanin. As expected, all white animals were pheomelanic (A650/500 < 0.15) and all black animals were eumelanic (A650/500 > 0.25). The brown animals were not uniform in melanin composition. There were four brown animals with A650/500 values between 0.15 and 0.25, indicating that their fibre was mixed pheomelanin/eumelanin, and the remaining 11 brown animals had predominantly eumelanic fibre (A650/500 > 0.25). Four possible explanations exist for these predominantly eumelanic "brown" alpacas: 1) that they are "black bays", i.e. contain a mixture of black eumelanin and pheomelanin, where there is a high proportion of eumelanin to pheomelanin such that the eumelanin content partially masks the pheomelanin content, 2) that they are actually weakly pigmented blacks (variously described as "warm black" or "dark brown" by breeders), 3) that they

are true eumelanic browns, or 4) more than one of these phenotypes exists in the species. Therefore, it cannot be ruled-out that a eumelanic brown phenotype, caused by as-yet-unidentified *Tyrp1* mutations, or by mutations in another gene, exists in alpacas. The selection of brown animals for this study was targeted to increase the probability of finding eumelanic browns, therefore a more comprehensive study of the pigment content of brown alpacas is required in order to determine whether the chemical composition of the brown alpacas in this study is representative of the species in general.

Analysis of phenotypic variants of genes is often targeted at mutations that alter the coding sequence of a gene. However, mutations exist within non-coding regions that have a regulatory effect on *Tyrp1* gene activity and pigment synthesis. Examples in other species include a *Tyrp1* intron 6 variant that disrupts the donor splice site, which has been associated with the chocolate phenotype in cats (Lyons et al., 2004, Schmidt-Küntzel et al., 2005). Pigment studies in both mice and humans have reported the importance of regulatory elements located in the 3' UTR of *Tyrp1* that influence gene activity (Fang et al., 2002, Yasumoto et al., 1997). Seven polymorphisms were found in the intronic regions of alpaca *Tyrp1*, and these were all examined for their possible effect on phenotype. However no association was observed between any single SNP and the brown phenotype (Table 5.2 & 5.3). It should be noted that only a small proportion of the intronic regions were sequenced in this study, but they did include the splice acceptor/ donor and branch sites for each exon. Subsequent work investigating the melanic basis of alpaca fibre by Cransberg et al. (2013) reported that alpaca fibre samples classified as visually brown were in fact mixed melanin. The results of this work strengthen the argument that eumelanic brown does not exist in alpacas and supports the findings of this project.

5.5 Conclusion

Given the enzymatic activity of Tyrp1, which is targeted at the eumelanic pathway, and the number of species where *Tyrp1* polymorphisms have been shown to be associated with brown fibre colour, this gene was identified as a strong candidate for the brown phenotype in alpacas. However no association

between any SNPs in alpaca *Tyrp1* and brown fibre colour was found, supporting the hypothesis that *Tyrp1* is not the cause of brown fibre colour in alpacas.

While the results of this study support the hypothesis that *Tyrp1* is not the cause of brown fibre colour in alpacas it does not provide sufficient evidence to rule out the involvement of this gene entirely in alpaca brown fibre colour phenotypes. The Australian alpaca population is only a small sample of this species and consequently the evidence from this study is not sufficient to conclude that a mechanism for Tyrp1 brown does not exist in other populations. Additionally, the results of melanin content analysis were not able to rule out the existence of a eumelanic brown phenotype in the species, so it is possible that another gene or a regulatory region mutation is causative of eumelanic brown in alpacas. These results contribute to the understanding of the underlying genetics of fibre colour inheritance in alpacas.

6

The Agouti Signalling Protein and black fibre phenotypes in alpacas

ASIP is one of the key regulators of pigment synthesis in mammals, functioning as a biological on/off switch for melanin synthesis in combination with the activity of MC1R. This chapter details work in which the alpaca ASIP *gene was sequenced in order to identify and characterise polymorphisms associated with fibre colour.*

6.1 Introduction

MC1R and *ASIP* share an epistatic relationship, where a fully functioning receptor (MC1R) is required for the effect of the *ASIP* alleles to be expressed. If MC1R is non-functional it will mask the effect of ASIP, because an inactive receptor is incapable of producing eumelanin, regardless of the allele(s) present at the *ASIP* locus (Furumura et al., 1996, Hoekstra, 2006). Due to its close interaction with MC1R, information about ASIP is essential to gain an understanding of the genetic mechanisms controlling colour inheritance in alpacas, a view that is supported by the fact that alpacas exhibit phenotypes analogous to phenotypes controlled by *ASIP* in other species; for example black and bay in horses (Rieder, 2009, Rieder et al., 2001); and black & tan in dogs (Kerns et al., 2004, Berryere et al., 2005).

The *ASIP* gene consists of three coding exons, most often termed exons 2, 3 and 4 (Kanetsky et al., 2002, Lu et al., 1994, Willard et al., 1995, McNulty et al., 2005, Fontanesi et al., 2010b). Many mammals have no reported mutations within the *ASIP* coding region, and in most species the coding region is highly conserved (Chen et al., 1996, Fontanesi et al., 2010b, Grapgodatskaya et al., 2002, McNulty et al., 2005, Royo et al., 2005, Siracusa, 1994). However, non-agouti black in dogs is the result of an *ASIP* Exon 4 mutation (Kerns et al., 2004).

The *Agouti* gene has a complex structure, including regulatory elements that affect gene expression, with several studies determining that most colour variation attributed to the *ASIP* gene is the result of these non-coding regions (Dinulescu and Cone, 2000, Fontanesi et al., 2010b). These regulatory elements are in the 5' UTR of *ASIP* and have been identified in a range of mammals including mice (Vrieling et al., 1994), rabbits (Fontanesi et al., 2010b, Vrieling et al., 1994), dogs (Kerns et al., 2004, Vage et al., 1997) and cattle (Girardot et al., 2004, Girardot et al., 2006). Transcripts specific for pigmentation on both dorsal and ventral surfaces have been identified, which are associated with different promoters, resulting in differential expression of the gene in different regions of the same animal (Vrieling et al., 1994, Hustad et al., 1995, Fontanesi et al., 2010b). Region specific expression of ASIP has been linked to certain

phenotypes such as the white-bellied agouti phenotype in mice where pheomelanin pigment is confined to the ventral surfaces (Bultman et al., 1994). Hair cycle-specific transcripts have also been identified, which switch the gene on and off during the growth cycle of a single fibre and results in the classic agouti phenotype of a pheomelanic band separating two eumelanic bands of pigment in a single fibre (Hustad et al., 1995, Siracusa, 1994, Vrieling et al., 1994). This phenotype is not seen in all species, but may be functioning in alpacas (cf. personal observation of banded hairs in a dam and her cria).

6.2 Materials and Methods

6.2.1 Sequencing of alpaca *ASIP*

Initial sequence analysis, using methods detailed in Chapter 2, was performed on 15 animals (three black, two black and tan, five brown, and five fawn). An additional 79 animals, comprising a wider range of colour phenotypes, were subsequently genotyped for Exon 4 variants.

6.2.2 Multiple alignment of ASIP sequences

A BLASTP (version 2.2.23) search was performed on UniProtKB (release 2010_10) with the Alpaca protein sequence as the query, an expectation threshold of 0.0001, Blosum62 scoring matrix, no filtering, and allowing gaps. The search resulted in 194 sequences, and 32 of these sequences were subsequently chosen for multiple sequence alignment (MSA). The 32 sequences were chosen according to four criteria: (1) only one species was represented by each sequence in the MSA, (2) the protein name was listed as 'Agouti Signalling Protein', (3) high expectation value, and (4) represented a range of sequence identities from 40% in Goldfish (Uniprot accession: Q5CC35) to 85% in pig (Uniprot accession: Q6ZYM3).

6.3 Results

6.3.1 Alpaca *ASIP* gene

The complete coding sequence of the alpaca *ASIP* gene was generated (GenBank accession no. HM768322). Similarity with the *ASIP* gene of other

species was confirmed, with the highest sequence similarity being to cow, goat and sheep (89%) and pig (88%). Exon 2 is 160 bp, exon 3 is 65 bp and exon 4 is 177 nucleotides in length. The entire coding region is 402 bp long, 6 bp longer than the dog and mouse, 3 bp longer than the human and 6 bp shorter than the cat *ASIP* coding region. A consensus splice acceptor and splice donor site flanks each coding exon. Exons 2, 3 and 4 contain 54, 40 and 68% GC (respectively) and the whole coding region is 58% GC.

6.3.2 Polymorphisms in alpaca *ASIP*

Sequencing of the *ASIP* coding region in 15 alpacas revealed five polymorphisms (Table 6.1). Two non-synonymous SNPs were identified in exon 4: c.C292T, predicted to cause an arginine-to-cysteine substitution at codon 98 (R98C; GenBank accession no. HM768323), and c.G353A, predicted to cause an arginine-to-histidine substitution at codon 118 (R118H; GenBank accession no. HQ008273). A 57 bp deletion was also discovered in exon 4. This deletion, occurring at nucleotides 325–381 (c.325_381del57; GenBank accession no. HM768324), is predicted to result in 19 of the last 25 amino acids being absent from the mature protein (p.C109_R127del). In addition, two synonymous mutations; c.G102A in exon 2 (p.G34) and c.C291A in exon 4 (p.T291) were identified. Four haplotypes involving the exon 4 mutations were observed in this group of alpacas, and these haplotypes existed in ten different combinations (Table 6.2). While no match occurred between any fibre colour and any single *ASIP* genotype in the 94 animals studied there was strong association between the homozygous state of each exon 4 non-synonymous mutation and black colour, as well as between the heterozygotes of these and black colour (Fisher's exact test for c.325_381del57 P<0·001; for c.292C>T P=0·039; for c.353G>A P=0·024; and for all combined P<0·001). Forty-eight of the 53 black animals were either homozygous for C109_R127del (allele a^1), the T allele of R98C (a^2), the A allele of R118H (a^3) or were heterozygous for a combination of two of these mutations. However, 18 of the 41 non-black animals also exhibited some of these genotype combinations. These animals exhibited a range of *MC1R* genotypes (Table 6.3). An additional five SNPs were identified in the non-coding regions flanking the exons (Table 6.1).

Table 6.1 Polymorphisms detected in the alpaca *ASIP* gene

Polymorphism	Location	Amino Acid Effect
c. 102G>A	Exon 2	Synonymous
c. 291C>A	Exon 4	Synonymous
Exon 3 +34C>T	Intron 2	N/A
Exon 3 +56A>C	Intron 2	N/A
Exon 4 -41C>A	Intron 2	N/A
c. 292C>T	Exon 4	R98C
c. 353G>A	Exon 4	R118H
c. 325_381del57	Exon 4	C109-R127del
Exon 4 +10C>T	3' UTR	N/A
Exon 4 +38A>G	3' UTR	N/A

Table 6.2 *ASIP* Genotypes of the three significant exon 4 polymorphisms examined

Putative *ASIP* Genotype	Exon 4 Genotype			Colour	No. of Animals
	c.292C>T	c.353G>A	c.325_381del57		
	R98C	R118H	C109_R127del		
a^1a^1	CC	-	Yes	Black Brown Fawn	21 3 1
a^2a^2	TT	GG	No	Black	4
a^3a^3	CC	AA	No	Black Brown White	5 2 1
AA	CC	GG	No	Fawn White	1 5
a^2a^3	CT	GA	No	Black Brown Fawn White	3 1 1 2
Aa^3	CC	GA	No	Fawn White	5 3

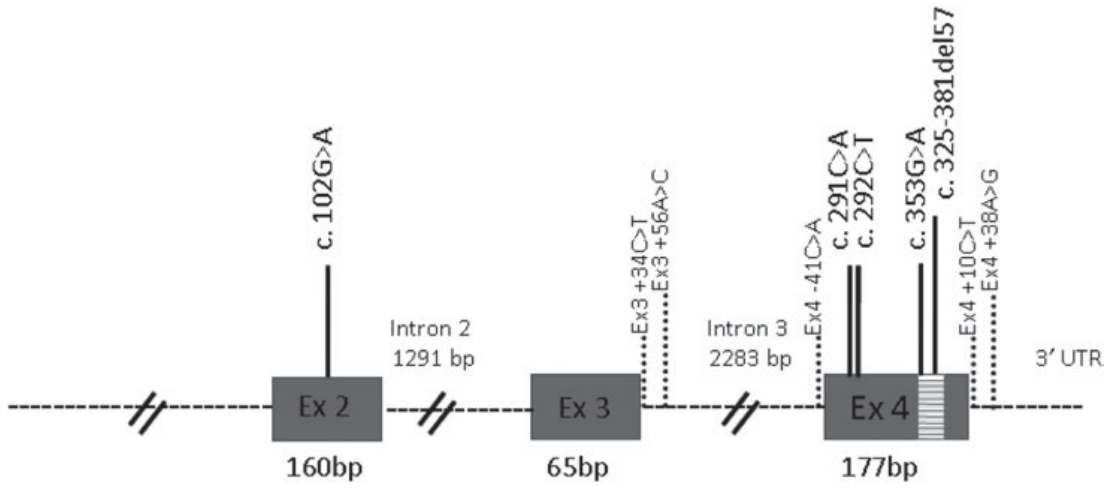
Aa²	CT	GG	No	Black Brown Fawn White	1 1 1 1
Aa¹	CC	G	Het	Black Silvergrey Brown Fawn White	4 1 2 2 1
a¹a³	CC	A	Het	Black Brown	2 2
a¹a²	CT	G	Het	Black Black & Tan Brown Fawn White	13 1 2 1 1

Table 6.3 *MC1R* Genotypes of non-black animals with *aa* genotypes

Putative *ASIP* Genotype	Fibre Colour	No. of Animals	*MC1R* Genotype
a¹a¹	Brown	3	*EE, Ee, ee*
	Fawn	1	*ee*
a³a³	Brown	2	*Ee ee*
	White	1	*ee*
a²a³	Brown	1	*Ee*
	Fawn	1	*Ee*
	White	2	*Ee Ee*
a¹a³	Brown	2	*Ee ee*
a¹a²	Black & Tan	1	*Ee*
	Brown	2	*Ee ee*
	Fawn	1	*ee*
	White	1	*EE*

6.3.3 Predicted alpaca ASIP protein

Alpaca *ASIP* translates into a predicted 133 amino acid protein (Fig. 6.1) that is 83% identical to sheep and cow ASIP and 81% identical to the horse and rabbit ASIP. The consensus prediction for the signal peptide in ASIP was from residues 1 to 22 by two out of three signal peptide prediction methods. The predicted peptide cleavage site was between residues 22(S) and 23(H). In contrast, Phobius predicted a signal peptide cleavage site between residues 24 and 25. A pairwise alignment of the alpaca ASIP sequence with the sequence of an engineered ASIP structure (Protein Data Base ID: 2KZA) showed 74% identity between the 53 residue, cysteine rich, C-terminal sequence. All cysteines in the pairwise alignment were exactly aligned. Consequently, it can be inferred by sequence similarity that the disulphide bonds in alpaca ASIP are likely to occur at cysteine residue locations [94–109], [101–115], [108–126], [112–133] and [117–124]. The disulphide bond prediction method DBCP (Lin & Tseng 2010) also predicted disulphide bonds at exactly the same locations.



(b)

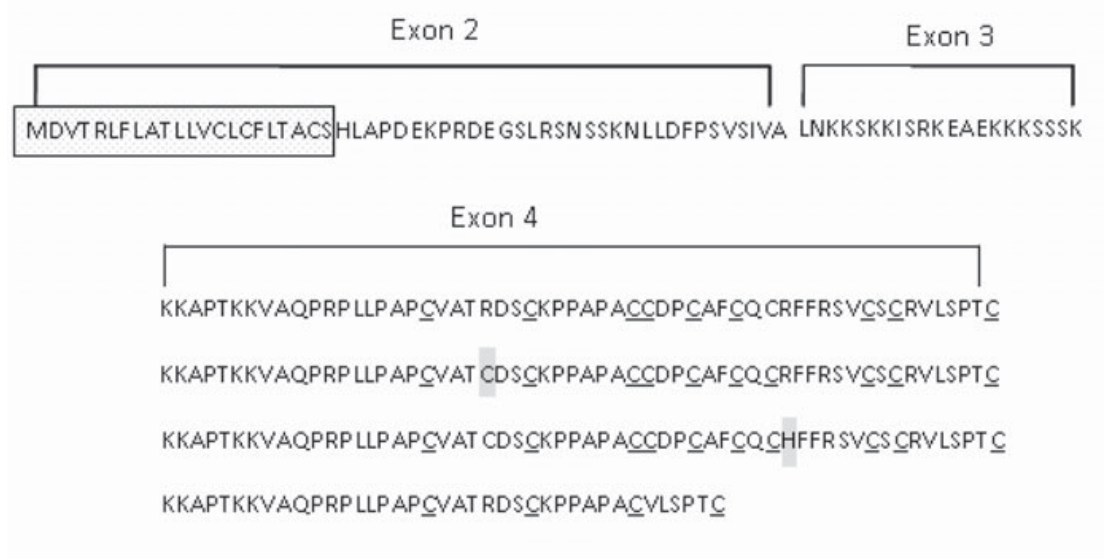


Fig. 6.1. The alpaca *ASIP* gene. (a) Coding exons 2, 3 and 4 are shown as solid boxes. Untranslated regions are shown as dashed lines (not to scale). Polymorphisms are shown in the regions where they occur. Indicated intron size was determined from the Ensembl genome assembly (b) The predicted alpaca wild-type ASIP protein. The conserved cysteine residues in the C-terminus are underlined; the secretion signal is shown as a dotted box. Predicted exon 4 protein sequences for the R98C, R118H (grey boxes) and C109_R127del mutations are shown below the wild type protein.

6.3.4 Polymorphisms analysis

The arginine residues at positions 98 and 118 in the alpaca ASIP sequence are highly conserved in all ASIP sequences aligned from 32 different mammalian species (Figure 6.1). All amino acid substitution prediction methods used

predicted that the p.R98C and p.R118H amino acid substitutions are potentially damaging to ASIP structure or function (Table 6.4). Only the SIFT method labelled its prediction as 'low confidence'. Therefore, it is proposed that the mutations p.R98C, p.R118H and p.C109-R127del are all loss of function non-agouti equivalent 'a' mutations for alpaca *ASIP*.

Table 6.4 Potential effect of amino acid substitution on ASIP structure or function. *The terms 'destabilizing', 'probably damaging' and 'possibly damaging' are used by the respective methods to describe the effect of the amino acid substitution.

Amino Acid Substitution Prediction Method	Amino Acid Substitution	
	R98C	R118H
SIFT	Likely to affect function (*low confidence*)	Likely to affect function (*low confidence*)
iMutant 2.0	Decrease in stability of structure (*High confidence*)	Decrease in stability of structure (*High confidence*)
iPTree-STAB	Destabilizing* to structure	Destabilizing to structure
PolyPhen	Probably damaging to structure and function (*high confidence*)	Possibly damaging to structure and function (*medium confidence*)

6.4 Discussion

Three novel variants in exon 4 of the alpaca *ASIP* gene have been identified and each is predicted to cause a loss of function in the protein. Exon 4 codes for the last 40 amino acid residues of ASIP, which includes the C-terminal domain (McNulty et al., 2005). The majority of protein and receptor binding activity directly involves this region (Dinulescu and Cone, 2000, Miltenberger et al., 2002).

ASIP displays some structurally distinctive characteristics. It has been reported that the ten cysteine residues within the C-terminus are involved in a network of five disulphide bonds that act to stabilize the protein (Willard et al., 1995, Yu and Millhauser, 2007, McNulty et al., 2005) and form a unique fold motif known as an inhibitor cysteine knot (ICK; McNulty *et al.* 2005; Yu & Millhauser 2007). The

spacing of these cysteine residues is strictly conserved throughout all mammals that have been investigated and this unique fold structure allows the presentation of three important conserved residues, Arg116Phe117Phe118, in order to facilitate MC1R interaction and binding (Miltenberger et al., 2002). This structure has been highly characterized through both chemical mapping and Nuclear magnetic resonance spectroscopy studies in the very similar Agouti related protein, and recent studies have confirmed the existence of the ICK fold in ASIP (Yu and Millhauser, 2007).

Much of the research examining the activity of ASIP reports that mutations in the C-terminal region most often severely impair or destroy ASIP function (Miltenberger et al., 2002, Yu and Millhauser, 2007). This structure can therefore be assumed to be essential for correct ASIP functioning. Animals carrying the deletion (aka a^1) are missing six of these ten conserved cysteine residues. This would almost certainly prevent tertiary structures, such as the ICK, from being formed correctly. With this type of disruption to the protein it is almost certain that function is eliminated.

The mutation found in exon 2 is a synonymous SNP resulting in no predicted structural changes, and it was polymorphic in every colour group represented in the current sample set. Therefore, although it occurs in the region that codes for the signal sequence and part of the lysine-rich basic region (Hustad et al., 1995, Willard et al., 1995), it is almost certainly only a population polymorphism.

About half of all known disease-causing mutations result from amino acid substitutions, and automated prediction methods can be used to identify potentially damaging substitutions (Ng and Henikoff, 2006). Four methods were used to derive a consensus view on the potential effect of the two observed amino acid substitutions on ASIP structure and function. It is preferable to use more than one prediction method, based on different assumptions and algorithms, because this gives increased support for any inference that may be made on the functional or structural significance of an amino acid substitution. In all cases the automated methods predicted a potentially damaging or destabilizing effect on ASIP structure or function for both SNPs (Table 6.4). Only the SIFT method labelled its prediction as ‘low confidence’. The SIFT method

depends on sequence diversity to generate confidence values and there was insufficient sequence diversity in the sequence alignment generated by SIFT; which uses PsiBlast to find and align functionally related sequences (Kumar et al., 2009, Ng and Henikoff, 2001). This is a common issue with prediction algorithms that depend on sequence conservation and is understandable given the highly conserved nature of the ASIP sequence.

There are a number of ways in which these SNPs might cause a loss of function in ASIP. The R98C polymorphism results in the substitution of a cysteine for a arginine in the highly conserved C-terminus of the protein (Willard et al., 1995, Bultman et al., 1992, Yu and Millhauser, 2007). Molecular analysis of the mouse agouti gene determined that these highly conserved cysteine residues are crucial for activity of the protein (Miltenberger et al., 2002, Perry et al., 1995). The addition of another cysteine residue in this region is predicted to disrupt the formation of this essential tertiary structure (Table 6.4) and be responsible for a loss of function of the protein. Protein alignment results suggest that the R98C polymorphism in alpacas is the same as the R96C polymorphism present in dogs, due to the upstream difference of two amino acids between the two proteins. The R96C mutation in dogs has been shown to be a loss of function mutation resulting in non-agouti black (Kerns et al., 2004). The similarity between these polymorphisms further supports the current *in silico* predictions that R98C has a loss of function affect in alpacas.

The residues Arg116Phe117Phe118 have an essential role in protein interactions of the C-terminal domain. When correctly folded, these are the binding determinants of the protein and they facilitate direct interaction with Melanocortin receptors (McNulty et al., 2005, Miltenberger et al., 2002). This suggests that the amino acid sequence of this domain and the correct structural folds are important determinants for protein function. The p.R118H substitution is usually considered a conservative substitution according to the Blosum62 evolutionary matrix (Henikoff and Henikoff, 1992) and this is understandable given the similar physicochemical properties of arginine and histidine.

However, any change in a highly conserved region has the potential to be

detrimental to a protein's structure or function. The conserved Arg116Phe117Phe118 amino acids are homologous to residues Arg118Phe119Phe120 in alpaca ASIP. Therefore, the p.R118H mutation results in a His-Phe-Phe combination in the active loop (instead of the conserved Arg-Phe-Phe combination), which may prevent ASIP interaction with MC1R, resulting in a loss-of-function variant. However, functional studies, such as testing the effect of these proteins on receptor signalling *in vitro*, or RNAi in mouse models, are needed to confirm that these mutations are affecting ASIP function.

It is interesting to note that the three exon 4 mutations probably occurred in historically independent populations. The p.R118H mutation cannot exist in a haplotype with p.C109_T127del19, and the T allele of p.R98C was never found in a haplotype with the deletion, nor with the A allele of p.R118H. Only four haplotypes were present in the sample of alpacas used in the current study: C,G,No, C,Yes, T,G,No and C,A,No. Black alpacas are considered to be sacred in South America, and are still sacrificed to the Gods (Bolin, 1998; J. C. Wheeler, personal communication). Therefore, historically, selective breeding would have preserved any new mutations causing black animals to occur.

It would be expected that animals that are homozygous for any of the non-functional *ASIP* alleles would have a black phenotype. This is largely consistent with the current data (Table 6.2): 48 out of 53 black animals were homozygous for a^1, a^2 *or* a^3, or were heterozygous for two of these alleles. The five animals that were black in phenotype but did not carry two putative black alleles could have regulatory mutations leading to decreased ASIP expression, or mutations at another gene (e.g. MC1R or β-defensin).

Almost half of the non-black animals were homozygous for the putative non-functional mutations. There are four reasons why non-black animals might carry a black genotype. The first is that a non-functional *MC1R* genotype is also present (i.e. *ee*), thus the specific *ASIP* allele is irrelevant. MC1R and ASIP have an epistatic relationship where a fully functioning MC1R receptor is necessary for the ASIP alleles to be expressed (Furumura et al., 1996, Hoekstra, 2006). If MC1R is non-functional then it cannot be activated or inactivated by either of the

two alternate ligands. It will therefore only express pheomelanin, and the *ASIP* alleles will be masked. Just over one-third of the non-black '*aa*' animals in this study are homozygous for the putative non-functional MC1R allele '*e*' (Table 5; Feeley & Munyard 2009); however, others were not. Secondly, if α-MSH is also non-functional, then MC1R will receive no signal from either its agonist or antagonist, and will revert to pheomelanin production.

A third possible explanation is that dilution genes are having an effect on the underlying colour. The dilution genes *MATP* and *TYR* have been investigated in alpacas, but no mutations were found that were linked with colour variation (Cransberg and Munyard, 2011). The mutation responsible for the Champagne dilution in horses has been identified in *SLC36A1* (Cook et al., 2008). This mutation renders black animals brown, and chestnut animals pale golden. Another possible candidate dilution gene is *Tyrp1*, which is known to change black eumelanin to brown eumelanin in many species (e.g. mice Zdarsky *et al.* 1990; dogs: Schmutz *et al.* 2002; cows: Berryere *et al.* 2003; sheep: Gratten *et al.* 2007). However results of experiments previously described in Chapter 5 of this thesis indicate that *Tyrp1* is not responsible for black/brown colour dilution in the Australian alpaca population.

The fourth potential reason why *'aa'* alpacas might be non-black is also a reason why some black animals are not *'aa'*: subjective phenotype assignment. There is considerable variation in the words used to describe similarly coloured alpacas. For instance, one breeder would call a faded black animal brown, while another would call it black. Similar problems also occur when assigning colours to paler animals. It is imperative that the industry adopts an objective way to measure phenotype, perhaps following the simple melanin analysis methods developed by Ito *et al.* (2000).

While putative black alleles have been identified in the coding regions of the alpaca *ASIP* gene, there is almost certainly additional variation in the regulatory regions of alpaca *ASIP* that leads to other phenotypes in this species. Molecular characterisation of ASIP and RNA expression studies in other species describe sequences upstream of the coding region termed untranslated exons

(Drogemuller et al., 2006, Vrieling et al., 1994, Hustad et al., 1995, Fontanesi et al., 2010b, Bultman et al., 1992, Kwon et al., 1994, Girardot et al., 2006). Different combinations of these exons are correlated with region specific and hair cycle expression although the mechanisms modulating the use of these untranslated exons is not well understood. In mice (Vrieling et al., 1994, Bultman et al., 1992) and rabbits there are four untranslated exons (Fontanesi et al., 2010b) and five in pigs and cattle (Drogemuller et al., 2006, Girardot et al., 2006). There is similarity between the untranslated exons among species though not all species contain the same number or same variants of exons. Pigs and mice for example both contain the adjacent 1A and 1A' exons located approximately 80kb upstream of the exon 2 start codon. But of the hair cycle specific exons termed 1B and 1C in mice, pigs contain only one exon showing similarity to the murine exon 1B, and exon 1C appears to be absent (Drogemuller et al., 2006, Vrieling et al., 1994)

Royo *et al.* (2008) demonstrated that the regulatory exons of *ASIP* are important in controlling ASIP expression in different breeds of sheep. Norris & Whan (2008) found that the white phenotype in sheep is dependent on a partial duplication of the regulatory region of *ASIP*, while Drogemuller *et al.* (2006) link the black and tan phenotype of pigs to a ventral specific regulatory mutation of *ASIP*. Alpacas with phenotypes resembling black and tan dogs and bay horses are common, and samples of alpaca fibre banded in a hair-cycle specific manner have been observed.

6.5 Conclusion

The three novel loss-of-function ASIP mutations identified in the current study can be used by alpaca breeders to decrease the frequency of black animals in their herd, by selecting against animals carrying any of the mutations. It will also allow breeders of black alpacas to retain the desired colour, increase their gene pool, and potentially improve the quality of fibre, by identifying high-quality non-black animals that carry black alleles. However, these mutations neither explain all instances of black nor all the phenotypic variation that is possible in *ASIP*.

7

General Discussion

The general hypothesis of this thesis was that *MC1R* and *ASIP* were the major determinants of normal colour variation of melanogenesis in alpacas. This hypothesis was supported by the results of this study, which established that alpaca fibre colour determination is controlled in much the same way as other mammalian colour models. It is predominately controlled by the interaction of *MC1R* and *ASIP*, which determine the pigment type produced, and form the basis of coat colour.

The main aims of this thesis, outlined in Chapter One, have been achieved with the following findings.

1) Characterise the *MC1R*, *ASIP* and *Tyrp1* genes in alpacas

Full sequencing of coding regions of the *MC1R*, *ASIP* and *Tyrp1* genes was performed on DNA samples collected from Australian alpacas. The exon structure of each gene was confirmed and this study reported 8 polymorphisms within *MC1R* and 4 within the coding region of *ASIP* in alpacas. The *Tyrp1* gene contained 11 novel polymorphisms within the coding region though only one resulted in an amino acid change.

2) Determine the relationships between polymorphisms in these pigment genes and the phenotypic variation observed in Australian alpaca populations

This study identified a wild type *MC1R* allele and two predicted non-functional alleles, R301C and S85X, which result in pheomelanic pigment. Also identified were three non-functional *ASIP* alleles, R98C, R118H and C109_R127del, which were associated with eumelanic fibre phenotypes. These polymorphisms explain some, but not all of the variation in the type of pigment produced in alpacas.

3) Determine if eumelanic brown exists in alpacas and investigate if *Tyrp1* is the cause of the 'visual brown' phenotype observed in alpacas

My work is evidence that *Tyrp1* brown phenotype may not exist in the Australian alpaca population. *Tyrp1* is responsible for eumelanic brown in some mammalian species but it is known that in some species the eumelanic brown phenotype does not exist. In reviewing this study an alternative hypothesis is

proposed in that eumelanic brown likely does not exist in alpacas and that the reported brown phenotypes are a mix of eumelanin/pheomelanin. Establishing this will be important as if it is confirmed that the 'brown' phenotype in alpacas is not a eumelanic variation but rather intensely pigmentation pheomelanic animals as suggested by this study, it will change the approach taken when using these animals to breed for a desired fibre colour. It is important to note that these conclusions about *Tyrp1* brown can only confidently be applied to the Australian alpaca population. The sample population examined in this study was not exhaustive and it is possible that *Tyrp1* allele may exist in other populations in the world, although rarely.

My work identified similarities in the mechanisms controlling fibre colour determination in other mammalian species. I identified two polymorphisms that were identical to phenotype affecting polymorphisms reported in dogs. These were the R98C polymorphisms in alpacas, homologous to the R96C reported in canines (Kerns et al., 2004), and the R301C polymorphism in MC1R. All other polymorphisms associated with fibre colour variation were unique to the alpaca but occurring within the same genes as other mammalian species. This is similar to what previous colour studies report, where the differences in colour determination between mammalian species are not in the pigmentation pathways but lay within the specific polymorphisms in genes within these pathways. As is commonly reported in mammalian species, the alpaca *MC1R* was found to be a highly polymorphic gene evidenced by the 22 SNPs found within a <1kb exon. This is a similar scenario to MC1R in other mammalian species, in which it is also reported to be an unusually polymorphic gene.

This study reports several polymorphisms in alpaca fibre colour genes. Since the commencement of this study other research groups have reported identical polymorphisms as well as additional ones not found in my sample group (Chandramohan et al., 2013, Powell et al., 2008, Valbonesi et al., 2011). However this was the first work to identify these polymorphisms as causal mutations of fibre colour variation of *MC1R* and *ASIP* in alpacas.

This study did not fully examine the regulatory regions of *MC1R*, *ASIP* or *Tyrp1* which may have helped to identify additional alleles for both black and white phenotypes as well as helping to explain additional fibre colour variations and phenotype patterns not explained by the current work. It is particularly likely that there is variation in the regulatory regions of *ASIP*, which are contributing to the black phenotype in alpacas. The role of regulatory regions of *ASIP* in pigmentation patterning and region and cycle specific expression is well documented in mammalian species. These include the agouti phenotype observed in mice (Bultman et al., 1992, Chen et al., 1996, Vrieling et al., 1994) and the black and tan colour pattern characteristic of the Doberman (Dreger and Schmutz, 2011).

One aspect of alpaca fibre colour that could not be explained by the results of this study is the variation in colour intensity observed between animals with the same genotype. As was presented in chapter three, animals carrying the homozygous loss of function C301 polymorphism ranged in colour from white through to fawn. While all these animals carried the 301C polymorphism and were not capable of producing eumelanin, pheomelanin was present in varying intensities. This variation may be a result of dilution genes. Dilution genes in mammalian pigmentation are well documented (Thiruvenkadan et al., 2008, Mariat et al., 2003, Cook et al., 2008, Brunberg et al., 2006, Schmutz and Berryere, 2007). A dilution effect is thought to be occurring in alpacas because of the wide range of fibre colour phenotypes they exhibit, and breeding outcomes in which parents produce offspring a lighter shade than themselves.

The membrane associated transport protein gene (*MATP*) is a good example of a pigmentation gene that has been established as having a dilution effect on both pheomelanic and eumelanic pigment in horses (Mariat et al., 2003, Locke et al., 2001). When either a chestnut or bay horse carries one copy of the defective *MATP* gene it results in the palomino and buckskin phenotypes, If two copies of the defective gene are inherited the chestnut animal is further diluted to a creamy white termed cremello while the bay animals are diluted to perlino. This difference in the amount of melanin is a comparable situation to that observed in this study where animals with the same *MC1R* genotype show varied intensities

of colour. The effect of *MATP* on horse pigmentation could be a possible explanation for the colour variation seen between alpacas in this study. *MATP* has also been reported as the cause of colour dilution in "underwhite" mice (Sweet et al., 1998) and oculocutaneous albinism type 4 in humans (Newton et al., 2001).

Colour variation in eumelanic animals was less identifiable. For example, the subtler difference in shade between what alpaca breeders classify a "warm black" and a "true black". If mutations in the coding region of suspected dilution genes are not responsible then the next alternative would be to investigate regulatory changes that affect gene expression. Such changes may have a more subtle effect on pigmentation than loss or gain of function coding mutations and may account for the variation observed between dark fibre animals. The paler fibre colour pattern of beach mice is a documented example of a regulatory change in a pigment gene that varies fibre colour (Steiner et al., 2007). Such changes often lead to an up regulation or down regulation of a particular gene's activity such as the *ASIP* variant in beach mice which results in increased agouti expression (Chen et al., 1996, Steiner et al., 2007). This type of regulatory change can results in an alteration in the amount of pigment rather than an all or nothing response like the loss of function mutations reported in both dog (Everts et al., 2000b) and horse MC1R (Markland et al., 1996) or the gain of function ASIP mutations in mice (Miller et al., 1993) in which pigment synthesis is restricted to producing only one type of melanin.

The subtle variation in eumelanic animals may also be a consequence of the structural difference between eumelanic and pheomelanic pigment. Eumelanin and pheomelanin differ in their sizes and shapes and inhabit the follicle differently. The difference between a "blue black" and a 'warm black" animal may be that one is a eumelanic black animal and the other is a combination of both pigment types in the one animal. Without further investigation it is difficult to pin point whether the variation in alpaca fibre colour phenotypes is due to as yet unknown underlying genetic traits or the environment or a mixture of both. As few as three alleles for a particular gene which result in slight differences in

colour intensity would produce 10 alternative genotypes in which the resulting phenotypes may appear visually as continuous variation.

Outcomes of the research

The biggest outcome of this work is that there is now a more comprehensive understanding of how colour inheritance is acting in alpacas. In addition to this the alpaca breeding industry now has the ability to test for known *ASIP* and *MC1R* variations, enabling them predict colour outcomes more accurately. Genetic tests for fibre colour to determine the colour breeding potential of animals are widely available in a number domesticated species, including horses and dogs, and this has led to well established breeding programs in these species (Rieder, 2009, UC Davis Veterinary Genetics Laboratory, 2013, Animal Genetics, 2014).

Alpaca fibre colour tests will be useful in maintaining genetic diversity in the alpaca population. Australian alpaca breeders wish to start creating commercial herds in an effort to increase production of fibre colours that are most valued on the market. However if you select for only one trait you run the risk of reducing the gene pool for other unknown traits. However, with a better understanding of the genes controlling fibre colour, quality animals of an undesirable colour may still be retained in the herd for breeding if they are known to carry alleles for the desired colour. For example, a white alpaca that exhibits fine fibre quality characteristics and carries black would previously have been disregarded by breeders interested in black fibre animals.

One of the strengths of this work was in the combined approach, which factored in the effect of genotype, phenotype and Mendelian inheritance patterns when analysing results. Mammalian coat colour is a complex and tightly regulated biological process involving the interaction of hundreds of genes, though a mutation in a single gene can be sufficient to alter the type of pigment produced (Markland et al., 1996, Ishida et al., 2006, Yu et al., 2013, Barsh, 1996). A study of alpaca coat colour and *MC1R* polymorphisms by Powell et al (2008) reported many of the same *MC1R* mutations as this study including C901T, which this study confirmed to be a non functional MC1R variant. However no correlation of

the C901T polymorphisms and loss of function of MC1R or pheomelanic pigment was reported by Powell et al. (2008) and this is likely a result of the method of phenotype assignment of the animals employed by that study.

The unique phenotype assignment that was pivotal in the investigation of *MC1R* was based on the alpaca coat colour theory proposed by Elizabeth Paul (Paul, 2006). Many coat colour investigations focus only on the fibre colour of the animals ignoring patterns and skin colour occurring in the animal. Paul (2006) proposed that black skinned white and pink skinned white alpacas were genetically distinct. While both have a white fibre colour phenotype, black skinned white animals were producing eumelanic pigment in the skin, whereas pink skinned white animals were not. When this selection criteria was applied to the white animals a pattern appeared that indicated that C901T was a likely causative polymorphism for pheomelanic animals. This study highlights the importance of the way in which data is interpreted. Analysing the animals as either having or lacking dark pigment (i.e. classing animals based the type of pigment present as opposed to focusing on visually ambiguous descriptions of colour phenotype) allowed for the identification of correlations between polymorphisms and coat colour that were previously overlooked. This example provided strong evidence for the need for a common nomenclature for phenotype classifications in alpacas that is genetically based.

Prior to the commencement of this study little was known about the genetic control of coat colour in alpacas and there was no universally accepted colour theory that fully explained all colour variations and patterns observed in alpacas. The process of selectively breeding animals in an effort to produce progeny of particular fibre colour phenotypes was well underway in the alpaca industry though the majority of theories were combined with unreliable breeding records making the process less effective. The work reported in this thesis has resulted in a more comprehensive theory of coat colour inheritance in alpacas. The molecular component of this work supported the proposed colour series in alpacas reported by Munyard in 2011.

The model identifies two alleles for *MC1R* and four for *ASIP*, which has been substantiated by the molecular analysis of these genes performed as part of this research. The model also hypothesises alleles for known colour patterns in alpacas though those are hypothetical and based on what information was gained from investigating the inheritance of the base colour. While not yet complete this model is the most comprehensive to date. This model was created using a similar approach of considering phenotype, genotype and Mendelian inheritance patterns when hypothesising a comprehensive theory. This study also highlights the need for a common nomenclature for phenotype classifications in alpacas that is genetically based.

Recommendations

In review, it is obvious that further studies will be needed before the genetic basis of the full range of fibre colours and patterns exhibited in alpacas can be explained. Several polymorphisms have been found which determine the base colour of the animal but not enough information was gathered to accurately predict the intensity of the pigment produced. Functional assays to confirm protein activity and interactions and gene expression studies to investigate the expression level of pigmentation genes would be valuable in uncovering additional genetic variation that is causative of phenotype variation, in particular the variation in pigment intensity observed among animals with identical *MC1R* genotypes. Many of the genes involved in mammalian pigmentation have variation in regulatory regions of the gene that are associated with fibre colour variation.

It is also likely there are more alleles for *MC1R* and *ASIP* that were not identified within this study, and that may be identified if a larger population of animals is sampled. This is evidenced by several research groups confirming identification of identical polymorphisms to this study in addition to reporting novel *MC1R/ASIP* polymorphisms (Chandramohan et al., 2013, Powell et al., 2008). While *MC1R* and *ASIP* were shown to be the major determinations of pigment type, *α-MSH* plays an important role in melanogenesis through ligand induced receptor activation. Future studies should include an analysis of *α-MSH* .

This work was the first to identify and characterise five novel polymorphisms in *MC1R* and *ASIP* that have been linked to specific fibre colour variations. Furthermore, the work reported in this thesis has added significant knowledge of the genetic determinants of fibre colour determination in alpacas. Further studies based on more extensive investigations of the regulation of pigment genes are expected reveal a more detailed understanding of alpaca fibre colour inheritance and lead to the availability of a greater number of fibre colour tests for breeding. The information reported in this thesis should provide significant benefits to the expanding alpaca fibre industry and assist in future colour breeding strategies.

8

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9

Appendix

Appendix 1A. GenBank Submissions for *MC1R*, *ASIP* and *Tyrp1* sequences identified in this study

Lama pacos melanocortin-1 receptor (MC1R) gene, complete cds
GenBank: EU135880.1

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gene, complete cds.
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VERSION     EU135880.1  GI:157165883
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SOURCE      Vicugna pacos (alpaca)
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Cetartiodactyla; Tylopoda;
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REFERENCE   1  (bases 1 to 1257)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Complete gDNA sequence of alpaca MC1R gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1257)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-AUG-2007) School of Biomedical
Sciences, Curtin
            University, GPO Box U1987, Perth, WA 6845,
Australia
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ORIGIN

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Lama pacos melanocortin-1 receptor (MC1R) gene, complete cds
GenBank: EU220010.1

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linear   MAM 28-OCT-2007
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gene, complete cds.
ACCESSION   EU220010
VERSION     EU220010.1  GI:158562210
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Vertebrata; Euteleostomi;
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REFERENCE   1  (bases 1 to 1336)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Complete gDNA sequence of alpaca MC1R
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1336)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-OCT-2007) School of Biomedical
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    241 gccaccaccc acctcggact ggccgccaac cagacggggc cccagtgcct ggaggtgtct
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Vicugna pacos isolate A123 tyrosinase-related protein 1 (TYRP1) gene, complete cds

GenBank: JN122622.1

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linear   MAM 06-NOV-2011
DEFINITION  Vicugna pacos isolate A123 tyrosinase-related
protein 1 (TYRP1)
            gene, complete cds.
ACCESSION   JN122622
VERSION     JN122622.1  GI:355918817
KEYWORDS    .
SOURCE      Vicugna pacos (alpaca)
  ORGANISM  Vicugna pacos
            Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria;
Cetartiodactyla; Tylopoda;
            Camelidae; Vicugna.
REFERENCE   1  (bases 1 to 18119)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Characterisation of the alpaca (Lama pacos)
TYRP1 gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 18119)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-JUN-2011) Biomedical Science,
Curtin University, Kent
            Street, Perth, WA 6845, Australia

FEATURES             Location/Qualifiers
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Lama pacos agouti signaling protein (ASIP) mRNA, complete cds

GenBank: HM768324.1

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linear   MAM 07-DEC-2010
DEFINITION  Lama pacos agouti signaling protein (ASIP)
mRNA, complete cds.
ACCESSION   HM768324
VERSION     HM768324.1  GI:312844249
KEYWORDS    .
SOURCE      Vicugna pacos (alpaca)
  ORGANISM  Vicugna pacos
            Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria;
Cetartiodactyla; Tylopoda;
            Camelidae; Vicugna.
REFERENCE   1  (bases 1 to 345)
  AUTHORS   Feeley,N., Bottomley,S. and Munyard,K.
  TITLE     Three novel mutations in ASIP associated with
black fibre in
            alpacas (Vicugna pacos)
  JOURNAL   J Agric Sci (2011) In press
REFERENCE   2  (bases 1 to 345)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JUL-2010) Curtin University,
Perth, WA 6845,
            Australia
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 241 aagaaggtgg cgcagccccg gcccctgctg cccgctccct gcgtggccac ccgcgacagt
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Lama pacos agouti signaling protein (ASIP) mRNA, complete cds
GenBank: HM768323.1

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LOCUS       HM768323                 402 bp    mRNA
linear   MAM 07-DEC-2010
DEFINITION  Lama pacos agouti signaling protein (ASIP)
mRNA, complete cds.
ACCESSION   HM768323
VERSION     HM768323.1  GI:312844247
KEYWORDS    .
SOURCE      Vicugna pacos (alpaca)
  ORGANISM  Vicugna pacos
            Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria;
Cetartiodactyla; Tylopoda;
            Camelidae; Vicugna.
REFERENCE   1  (bases 1 to 402)
  AUTHORS   Feeley,N., Bottomley,S. and Munyard,K.
  TITLE     Three novel mutations in ASIP associated with
black fibre in
            alpacas (Vicugna pacos)
  JOURNAL   J Agric Sci (2011) In press
REFERENCE   2  (bases 1 to 402)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JUL-2010) Curtin University,
Perth, WA 6845,
            Australia
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ORIGIN

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Lama pacos agouti signaling protein (ASIP) mRNA, complete cds
GenBank: HM768322.1

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linear   MAM 07-DEC-2010
DEFINITION  Lama pacos agouti signaling protein (ASIP)
mRNA, complete cds.
ACCESSION   HM768322
VERSION     HM768322.1  GI:312844245
KEYWORDS    .
SOURCE      Vicugna pacos (alpaca)
  ORGANISM  Vicugna pacos
            Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria;
Cetartiodactyla; Tylopoda;
            Camelidae; Vicugna.
REFERENCE   1  (bases 1 to 402)
  AUTHORS   Feeley,N., Bottomley,S. and Munyard,K.
  TITLE     Three novel mutations in ASIP associated with
black fibre in
            alpacas (Vicugna pacos)
  JOURNAL   J Agric Sci (2011) In press
REFERENCE   2  (bases 1 to 402)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JUL-2010) Curtin University,
Perth, WA 6845,
            Australia
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181 aagatcagca gaaaagaagc ggaaaagaag aaaagctctt ccaagaaaaa ggctccgacg
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Lama pacos agouti signaling protein (ASIP) mRNA, complete cds
GenBank: HQ008273.1

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linear   MAM 07-DEC-2010
DEFINITION  Lama pacos agouti signaling protein (ASIP)
mRNA, complete cds.
ACCESSION   HQ008273
VERSION     HQ008273.1  GI:312843883
KEYWORDS    .
SOURCE      Vicugna pacos (alpaca)
  ORGANISM  Vicugna pacos
            Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria;
Cetartiodactyla; Tylopoda;
            Camelidae; Vicugna.
REFERENCE   1  (bases 1 to 402)
  AUTHORS   Feeley,N., Bottomley,S. and Munyard,K.
  TITLE     Three novel mutations in ASIP associated with
black fibre in
            alpacas (Vicugna pacos)
  JOURNAL   J Agric Sci (2011) In press
REFERENCE   2  (bases 1 to 402)
  AUTHORS   Feeley,N.L. and Munyard,K.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-JUL-2010) School of Biomedical
Sciences, Curtin
            University, Kent Street, Bentley, Western
Australia 6102, Australia
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ORIGIN
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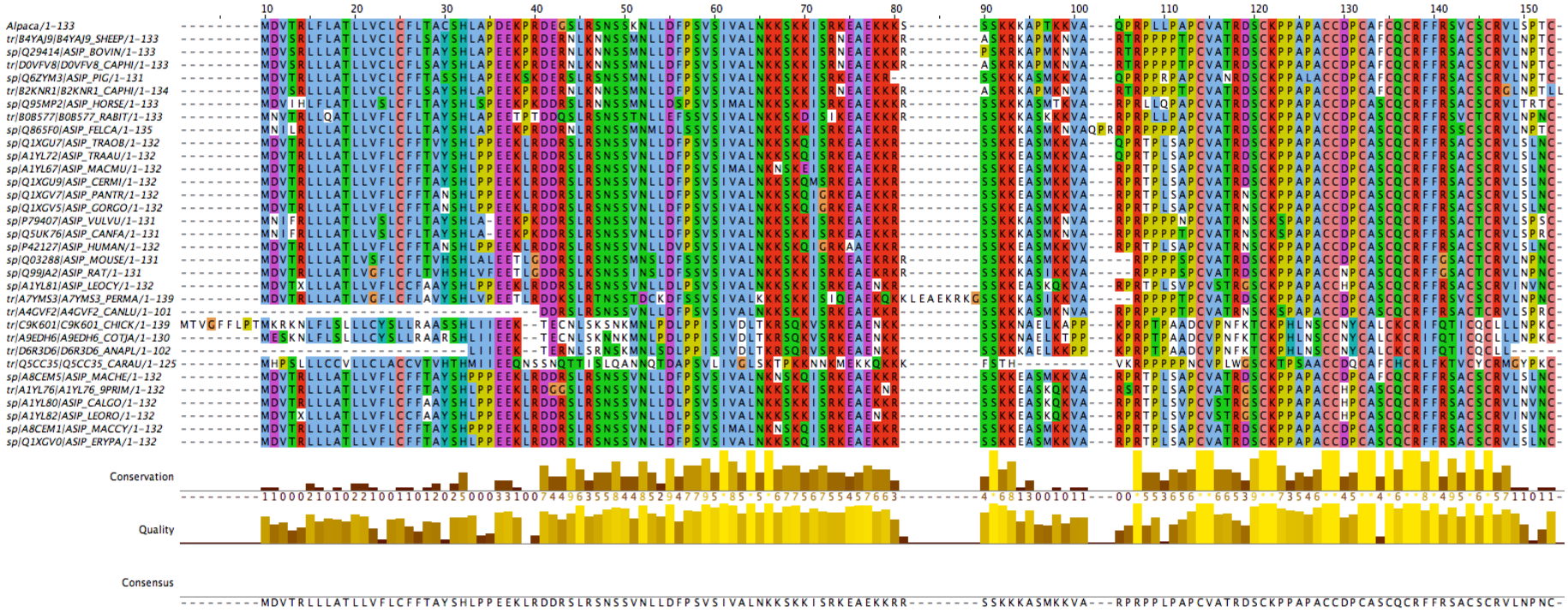
  1 atggatgtca cccgcctctt cctagctacc ctgctggtct gcctgtgctt cctcactgcc
 61 tgcagccacc tagcacctga cgaaaagccc agggatgaag ggagcctgag aagcaactcc
121 tccaagaacc tgttagattt cccttctgtc tctattgtgg cactgaacaa gaaatccaag
181 aagatcagca gaaaagaagc ggaaaagaag aaaagctctt ccaagaaaaa ggctccgacg
241 aagaaggtgg cgcagccccg gcccctgctg cccgctccct gcgtggccac acgcgacagt
301 tgcaagcctc ctgctcctgc ctgctgtgac ccgtgtgcct tctgccagtg ccacttcttc
361 cgcagcgtct gctcctgccg cgtgctcagc cccacctgtt ga
```

Appendix 1B. Multiple Sequence Alignment of 32 ASIP Protein sequences.



Appendix 1C.

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Characterisation of the melanocortin-1 receptor gene in alpaca and identification of possible markers associated with phenotypic variations in colour

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Abstract. The aim of this study was to determine if any correlation exists between melanocortin-1 receptor (*MC1R*) polymorphisms and skin and fibre colour in alpacas. Primers capable of amplifying the entire alpaca *MC1R* gene were designed from a comparative alignment of *Bos taurus* and *Mus musculus MC1R* gene sequences. The complete *MC1R* gene of 41 alpacas exhibiting a range of fibre colours, and which were sourced from farms across Australia, was sequenced from PCR products. Twenty-one single nucleotide polymorphisms were identified within *MC1R*. Two of these polymorphisms (A82G and C901T) have the potential to reduce eumelanin production by disrupting the activity of MC1R. No agreement was observed between fibre colour alone and *MC1R* genotype in the 41 animals in this study. However, when the animals were assigned to groups based on the presence or absence of eumelanin in their fibre and skin, only animals that had at least one allele with the A82/C901 combination expressed eumelanin. We propose that A82/C901 is the wild-type dominant '*E*' *MC1R* allele, while alpacas with either G82/T901 or G82/Y901 are homozygous for the recessive '*e*' *MC1R* allele and are therefore unable to produce eumelanin.

Additional keywords: melanin, pigmentation, single nucleotide polymorphism.

Introduction

The price and desirability of alpaca fibre is governed by characteristics including fibre type, length, diameter, evenness, yield and colour (Fleet *et al*. 1995; Frank *et al*. 2006). Colour is an important fibre characteristic because of its influence on the potential applications of the end product. There is currently no definitive model to explain the mechanisms that govern fibre colour inheritance in alpacas, therefore breeding programs are not always efficient in selecting for colour (McGregor 2006). Current colour breeding strategies use the fibre colour of the parents to predict breeding outcomes (Paul 2006). However, phenotype can be a poor indicator of genotype because of the complex nature of pigment regulation in mammals (Rees 2003; Hoekstra 2006). Fibre phenotype is the outcome of the combined effect of numerous genes, which makes it difficult to determine which genes are responsible for fibre colour when only relying on phenotype alone as an indicator (Furumura *et al*. 1996; Sponenberg 2001). Exactly which genes and alleles are important in alpaca fibre colour remains to be determined. The ability to determine the mechanisms of fibre colour inheritance in alpacas would be highly valuable to the industry because it would facilitate more precise selection and breeding for desired colours.

While alpaca fibre colour has been examined in regard to phenotype in several studies, there is a serious lack of information regarding the genetic control of fibre colour (Frank *et al*. 2006; McGregor 2006). Some of the specific work on fibre colour included analysis of the biochemical components of pigment in llamas, a closely related member of the Camelid family, and more recently, a study of an alpaca fibre colour gene in a selection of American alpacas (Cecchi *et al*. 2004, 2007; Powell *et al*. 2008). While all studies were an important addition to the current knowledge, none has provided enough information alone. Extensive research is still needed before selective breeding programs for colour can be effective.

Pigmentation mechanisms in mammals have been extensively studied and over 100 genes are known to be involved in the pigmentation process (Guibert *et al*. 2004; Hoekstra 2006; Ishida *et al*. 2006). These mechanisms are known to be highly conserved among mammals and therefore information from well-characterised species can serve as a suitable basis for predicting mechanisms involved in alpaca coat colour inheritance (Rees 2000, 2003; Hoekstra 2006). Mammalian coat colour is predominantly dependent on the amount, type and distribution of melanin in hair follicles (Furumura *et al*. 1996; Rees 2003). Mammals are capable of producing two chemically distinct melanin types known as eumelanin and pheomelanin, which are responsible for black to brown, and red to yellow pigment respectively (Smith *et al*. 2001; Oyehaug *et al*. 2002). While pigmentation is a complex process known to be influenced by a large number of genes, the primary regulation of pigment type is controlled by two key genes known as the melanocortin-1 receptor (*MC1R*) and *Agouti* (Sturm *et al*. 2001; Slominski *et al*. 2004; Rouzaud and Hearing 2005).

MC1R is a membrane-bound receptor expressed on the surface of melanocytes and has an integral role in pigmentation. It is

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responsible for initiation of several intracellular processes that determine whether pigment production will favour eumelanin or pheomelanin. These include activation of the adenyl cyclase pathway and controlling levels of a crucial enzyme in melanin production, tyrosinase (Hearing 2005; Hoekstra 2006; Tully 2007). The different alleles at the locus are responsible for either increasing or reducing the type of melanin produced (Busca and Ballotti 2000; Oyehaug *et al*. 2002; Thiruvenkadan *et al*. 2008). Gain of function alleles will extend the amount of eumelanin and reduce the amount of pheomelanin while loss of function alleles will increase pheomelanin and reduce eumelanin (Sturm *et al*. 2001; Hoekstra 2006; Thiruvenkadan *et al*. 2008).

The switch between the synthesis of these two pigment types is dependent on interactions of the receptor with either α-melanocyte stimulating hormone (MSH) or Agouti signalling protein. In the presence of α-MSH, the receptor is in its active conformation and is capable of initiating the essential cellular processes necessary for eumelanin synthesis, including cyclic AMP and tyrosinase production. However, if the protein product of the *Agouti* gene, Agouti signalling protein, is present it blocks receptor activation by α-MSH and induces pheomelanin production (Busca and Ballotti 2000; Oyehaug *et al*. 2002; Newton *et al*. 2005). A normally functioning melanocyte has the capacity to produce both melanin types; however, only one type is produced at a time (Thiruvenkadan *et al*. 2008).

MC1R is a G-protein coupled receptor (GPCR) (Scott *et al*. 2002; Newton *et al*. 2005). GPCR are an extensive family of cell surface proteins that display a common molecular structure consisting of five types of domains, an extracellular N-terminus, seven transmembrane fragments connected by three extracellular and three intracellular loops, and an intracellular C-terminus (Schoneberg *et al*. 2004; Sanchez-Mas *et al*. 2005; Tao 2006). These domains have roles that are vital for the functionality of the receptor and therefore polymorphisms located in important domains may affect pigmentation.

The association between polymorphisms within *MC1R* and pigment variation has been extensively studied and documented in a range of mammals including mice (Hoekstra *et al*. 2006), pigs (Kijas *et al*. 1998), cows (Rouzaud *et al*. 2000), dogs (Everts *et al*. 2000), foxes (Vage *et al*. 2005), horses (Marklund *et al*. 1996) and humans (Frandberg *et al*. 1998; Grimes *et al*. 2001). *MC1R* alleles that are functional mutations or activating mutations result in a receptor that is constantly active and that only synthesises eumelanin, the dark pigment (Barsh 1996; Carroll *et al*. 2005; Adan 2006). However, alleles that result in a non-functional receptor are characterised by a complete absence of black pigment (Everts *et al*. 2000; Oyehaug *et al*. 2002; Le Pape *et al*. 2008). The objective of this study was to characterise *MC1R* in alpacas and to investigate the association of polymorphisms within *MC1R* with fibre colour.

Materials and methods

Animals and DNA extraction

Blood samples were collected from 41 alpacas (36 Huacaya and five Suri). Initial sample analysis was carried out on nine entirely white and 14 entirely black animals. A second group of animals comprising a wider range of colour phenotypes were subsequently analysed (three black/brown, two grey, two dark brown, nine fawn, one rose/grey and one white animal). Fibre colour phenotype was determined according to a colour chart provided by the Australian Alpaca Association for colour registration. Samples were collected from animals bred in the states of Western Australia, New South Wales and Victoria, in Australia. Genomic DNA was extracted from 200 µL of EDTA-anticoagulated blood using the DNeasy tissue kit (Qiagen, Doncaster, Vic., Australia) according to the manufacturer's instructions.

Amplification, cloning and sequencing of alpaca MC1R

Alpaca *MC1R* primers MC1R1-F and MC1R2-R (Table 1) were designed to hybridise to conserved regions flanking the cow (*Bos taurus*) and mouse (*Mus musculus*) *MC1R* sequence (GenBank accession numbers NM_174108 and NM_008559), and were used to amplify the complete alpaca *MC1R* coding region.

All PCR were carried out in an Eppendorf Mastercycler (Eppendorf, North Ryde, NSW, Australia), in 10 µL reactions containing 67 mmol/L Tris.HCl (pH 8.8), 16.6 mmol/L $[NH_4]_2SO_4$, 0.45% v/v Triton X-100, 0.2 mg/mL gelatin, 0.2 mmol/L dNTP (Fisher Biotech, Wembley, WA, Australia), 2 mmol/L each of forward and reverse primer, 1 U *Taq* DNA polymerase (Fisher Biotech), 1.5 mmol/L $MgCl_2$ and 100 ng genomic DNA. Thermal cycles were initial denaturation at 95°C for 5 min, followed by 30 cycles, each consisting of 94°C for 30 s, 58°C for 30 s and 72°C for 1.5 min; with a final extension at 72°C for 10 min. Amplified DNA was analysed by electrophoresis in 1.5% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualised by UV transillumination.

PCR products from each animal were purified using the UltraClean PCR Clean-up Kit (Mo Bio, Carlsbad, CA, USA). DNA was cloned using the pGEMTeasy cloning kit (Promega, Alexandria, NSW, Australia), and each clone was sequenced using both M13-F and M13-R primers (Table 1) with Big Dye Terminator Technology (Applied Biosystems, Scoresby, Vic., Australia) and analysed on a 3730 DNA analyser (Applied Biosystems).

The sequences obtained from the two cloned PCR fragments were used to design alpaca-specific primers, MC1R3-F and MC1R4-R (Table 1), which were subsequently used to

Table 1. Primers used in this study
MC1R, melanocortin-1 receptor

Primer	Sequence
MC1R1-F	CTGGCACCACGGAGCTTG
MC1R2-R	CTCCTCAGCCTGCTCCATTC
MC1R3-F	GGGAGAAGGTGAGTGTGAGG
MC1R4-R	GCTCTTCCTGGAGATTCGTG
MC1R5-F	CATGGTGTCCAGCCTCTGCT
MC1R6-R	CCACCCAGATGGCCGCGATG
M13-F	CCCCAGGGTTTTCCCAGTCACGAC
M13-R	TCACACAGGAAACAGCTATGAC

amplify *MC1R* from 36 animals using the PCR conditions described above. Amplification of *MC1R* from each animal was carried out in five independent 10-μL reactions, which were pooled before product purification and sequencing. Sequencing reactions were carried out as described above except using four primers for each product: MC1R3-F and MC1R4-R, and two alpaca-specific internal primers, MC1R5-F and MC1R6-R (Table 1). Complete *MC1R* sequences for each animal were compiled into contigs using Vector NTI software (Invitrogen, Mount Waverley, Vic., Australia), and compared with genes and proteins from other species by GenBank NCBI BLASTn and BLASTx protocols (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Alpaca MC1R *sequence*

An NCBI BLAST search confirmed homology with *MC1R* of other species (GenBank accession number EU135880). Sequence similarity with alpaca was: pig and sheep 88%, goat 87%, cow 86%, human 85%, horse 84% and mouse 81%. Twenty-one single nucleotide polymorphisms (SNP) were identified within the *MC1R* coding regions in the 41 animals. However, only seven of the SNP were present in more than two animals (Table 2).

Translation of the *MC1R* sequence revealed an open reading frame of 317 amino acids. Four of the seven common SNP caused no amino acid change (D42D, N118N, L206L and E311E) while the remaining three resulted in amino acid substitutions (T28A, G126S and R301C). The alpaca MC1R protein was most similar to sheep MC1R (88%), followed by cow and cat (87%), pig (85%) and horse and dog (84%).

No correlation was observed between fibre colour alone and *MC1R* genotype in the 41 animals studied. However, when the animals were assigned to groups based on the presence or absence of eumelanin in fibre and skin, only animals that had at least one allele with the A82/C901 combination expressed eumelanin (Tables 2, 3). Animals that did not have an A82/C901 combination (i.e. were G82/T901 or G82/Y901 genotypes) expressed only pheomelanin (Tables 2, 3).

Discussion

Genotypes A82G and C126T were in complete concordance and hence are considered to be a haplotype. Analysis of results identified the G82/C126 combination as a possible marker for animals that had an absence of black pigment. These SNP were correlated with the presence or absence of eumelanin in skin and fibre. All pink-skinned white animals had the G82/C126 combination while all black animals were either heterozygotes or had the opposing combination A82/T126. The animals expressing pheomelanin only are hypothesised to have the genotype '*ee*' representing the homozygous recessive genotype at *MC1R*, while the eumelanic animals are proposed

Table 2. Phenotype and *MC1R* genotypes of the initial alpaca samples examined in this study

'*E*' denotes the proposed wild type allele and '*e*' denotes the proposed recessive alleles at melanocortin-1 receptor (*MC1R*). Single nucleotide polymorphisms (SNP) in bold are those that showed phenotypic correlations

SNP genotype							Fibre	Eumelanin	Proposed
82 (T28A)	126 (D42D)	354 (N118N)	376 (G126S)	618 (L206 L)	901 (R301C)	933 (E311E)	colour	present	*MC1R* alleles
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/A	**T/T**	A/A	White	No	*ee*
G/G	C/C	C/T	**G/G**	A/A	**T/C**	A/A	White	No	*ee*
G/G	C/C	C/C	**G/G**	A/G	**T/C**	A/G	White	No	*ee*
G/A	C/T	C/T	**G/A**	A/G	**T/C**	A/G	Black	Yes	*Ee*
G/A	C/T	C/T	**G/G**	A/G	**T/C**	A/G	Black	Yes	*Ee*
G/A	C/T	C/T	**G/A**	A/G	**C/C**	A/G	Black	Yes	*Ee*
A/A	T/T	T/T	**A/A**	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**G/A**	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	G/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**T/C**	A/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**C/C**	G/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**C/C**	A/G	Black	Yes	*EE*
A/A	T/T	T/T	**A/A**	A/G	**T/C**	A/G	Black	Yes	*EE*

Table 3. Colour phenotype and *MC1R* genotypes of the additional alpaca samples examined in this study at the three significant polymorphisms

'*E*' denotes the proposed wild type allele and '*e*' denotes the proposed recessive alleles at melanocortin-1 receptor (*MC1R*). Single nucleotide polymorphisms (SNP) in bold are those that showed phenotypic correlations. Indet., eumelanin status was indeterminable

SNP genotype			Fibre colour	Eumelanin present	Proposed *MC1R* alleles
82 (T28A)	126 (D42D)	901 (R301C)			
A/A	T/T	**C/C**	Fawn	Yes	*EE*
A/A	T/T	**C/C**	Fawn	Yes	*EE*
A/A	T/T	**C/C**	Fawn	Yes	*EE*
A/A	T/T	**C/C**	Silver/grey	Yes	*EE*
A/A	T/T	**C/C**	Dark brown	Yes	*EE*
A/A	T/T	**C/C**	Dark brown	Yes	*EE*
A/A	T/T	**C/C**	Medium grey	Yes	*EE*
A/G	T/C	**C/T**	Black/brown	Yes	*Ee*
A/G	T/C	**C/T**	Fawn	Yes	*Ee*
A/G	T/C	**C/T**	White	Yes	*Ee*
A/G	T/C	**C/T**	Black/brown	Yes	*Ee*
A/G	T/C	**C/T**	Fawn	Yes	*Ee*
A/G	T/C	**C/C**	Black/brown	Yes	*Ee*
G/G	C/C	**T/T**	Rose/grey	No	*ee*
G/G	C/C	**T/T**	Fawn	No	*ee*
G/G	C/C	**T/T**	Fawn	No	*ee*
G/G	C/C	**T/T**	Fawn	No	*ee*
G/G	C/C	**C/T**	Fawn	Indet.	*ee*

to have the genotypes '*EE*' (homozygous wild type) or '*Ee*', which both allow normal eumelanin expression.

Analysis of sequence data from a larger sample group (Table 3) correlated with what had been found previously in the initial sample group. Animals that had the G82/C126 combination were also those animals that had an absence of black pigment in skin and hair.

The A82G polymorphism occurs in the extracellular loop of the N-terminus of *MC1R* (Schioth *et al*. 2005; Tao 2006). Some residues in this domain have been shown to be important in normal functioning of the protein (Garcia-Borron *et al*. 2005). Alanine is a non-polar, hydrophobic amino acid while threonine is a polar hydrophilic amino acid (Stoker 2001). Substitution of alanine in place of threonine may alter the structure of the protein and inhibit its interactions and normal functioning; however, this cannot be confirmed with our results alone (Stoker 2001). The fact that the polymorphism displays perfect correlation with light pigment gives support to the argument that it is linked to phenotype variation.

The C126T polymorphism is synonymous so it is not likely that it has an independent phenotypic effect (Stoker 2001). It does, however, appear in a haplotype that displays perfect correlation with phenotype. While it is not possible from our results to definitively predict the phenotypic effect of the haplotype, it does appear that they have significance in relation to fibre colour variation. All animals with the G82/C126 combination were characterised by a lack of dark pigment. Animals that were either heterozygous or had the A82/T126 combination displayed dark pigment in skin and/or fibre. It is not clear from the information we have gained so far whether these mutations are causative of a change in phenotype or merely linked to the absence of black pigment. It may be possible that this SNP is linked to a promoter mutation and does not necessarily cause the phenotypic change (Hornyak *et al*. 2001; Smith *et al*. 2001; Rouzaud and Hearing 2005). These polymorphisms may serve as a good predictor of pheomelanic animals for breeding.

C901T also appeared to be a significant candidate polymorphism for phenotype effect. Animals with the A82/T126/C901 combination were capable of producing eumelanin while animals with the G82/C126/T901 combination lack any eumelanic pigment in skin or hair fibres. The C901T polymorphism occurs in the C-terminus of the protein and results in an arginine for cysteine substitution (Strader *et al*. 1994; Stoker 2001; Schioth *et al*. 2005). Exchanging an arginine for a cysteine may affect the structure of the *MC1R* C-terminus (Strader *et al*. 1994; Stoker 2001). Changing from a positively charged amino acid to a neutral amino acid could prevent proper interaction of the receptor with G-proteins and result in a non-functional receptor; however, determining if this is the case is particularly difficult given the region in which the polymorphism occurs (Strader *et al*. 1994; Stoker 2001; Tao 2006).

It is difficult to definitively determine the effect of this SNP on the functionality of MC1R without functional analysis of the alternative proteins. Many SNP affect protein function, yet structural modelling is difficult because of the inability of modelling programs to adequately detect the effect of a single SNP on protein structure and/or expression (Stitziel *et al*. 2003). This is especially the case where the polymorphism occurs in the C-terminal loop of the amino acid chain, which has a tendency to be flexible and difficult to model (Krystek *et al*. 2006).

C901T also occurs in an extremely significant domain in regard to structural integrity and function of the receptor (Strader *et al*. 1994; Tao 2006). The C-terminus in GPCR is a functionally important domain involved in interactions with the ligand–receptor complex with G-proteins, placement of the receptor within the membrane, and providing signals for intracellular trafficking (Schoneberg *et al*. 2004; Garcia-Borron *et al*. 2005; Sanchez-Mas *et al*. 2005). Polymorphisms in this domain are reported to impair receptor function severely (Everts *et al*. 2000; Garcia-Borron *et al*. 2005; Sanchez-Mas *et al*. 2005). If these interactions are not properly carried out, downstream processes essential for the production of eumelanin are not initiated, resulting in the default colour, pheomelanin, being produced (Newton *et al*. 2000; Logan *et al*. 2003; Hoekstra 2006). The C901T SNP appeared to be a likely candidate for affecting phenotype but it is not clear what effect it would have on the protein function and the receptor's ability to produce pigment.

However, the C901T SNP does not display perfect correlation with phenotype because there were heterozygote animals in both phenotype groups. If the homozygote T variant is reducing the ability of the receptor to properly function then it is possible that the heterozygous state may allow for partial functioning of the receptor (Jimenez-Cervantes *et al*. 2001; Beaumont *et al*. 2007). This may explain the presence of heterozygote animals in both the eumelanin-only and pheomelanin-only groups. The heterozygote animals would still have one functional copy of the gene, so it is possible this allows reduced production of eumelanin. This may

also explain variation between dark-fibred animals, as animals that were homozygous C would be able to produce pigment more effectively and hence possibly produce more pigment. Further functional analysis of this polymorphism would be necessary before any conclusions could be made.

The G376A polymorphism occuring at codon 126 is located in the central portion of the third transmembrane fragment (Strader *et al*. 1994; Garcia-Borron *et al*. 2005; Tao 2006). This results in the substitution of a non-polar glycine for a polar uncharged serine (Stoker 2001). It is possible that this type of substitution could alter the structure of the protein and affect its ability to function effectively (Strader *et al*. 1994; Jackson 1997; Hoekstra 2006; Tao 2006; Lin and Fisher 2007). There are several reported polymorphisms close to this position in the fox and pig *MC1R* that have been linked to phenotypic changes. Both are activating mutations resulting in dark fibre colour (Vage *et al*. 1997; Kijas *et al*. 1998). However, we found no correlation of any phenotypic trait with this polymorphism.

This study examined animals representing seven of over 20 recognised alpaca fibre colour phenotypes (Cecchi *et al*. 2004; Paul 2006) and both coat types (Suri and Huacaya). Determining correlations between polymorphisms and phenotype in alpacas is difficult because of the nature of pigment-related genes. There are over 100 known genes involved in the pigmentation process with the potential for colour to be affected at many stages (Rees 2003; Slominski *et al*. 2004). This is evident in our results as some animals with an identical *MC1R* genotype displayed different phenotypes (Tables 2, 3). It is probable that other genes are acting to modify coat colour in these animals (Jackson 1997; Hoekstra 2006; Lin and Fisher 2007). There are no pure breeding lines of alpacas that can be used to confirm phenotypes through test-cross breeding of animals, making it difficult to accurately determine the effect of identified polymorphisms. This is made even more difficult by the fact that alpaca breeders frequently cross animals of dissimilar colours.

The epistatic relationship between *MC1R* and the *Agouti* gene makes it especially difficult to make predictions on the effect of such *MC1R* polymorphisms without knowledge of the *Agouti* genotype (Furumura *et al*. 1996; Lin and Fisher 2007). *Agouti* is effective only in the presence of a fully functioning MC1R receptor and can cause a range of colours and patterns including completely eumelanic and completely pheomelanic (Rieder *et al*. 2001; Voisey *et al*. 2001; Kerns *et al*. 2003; Girardot *et al*. 2006). This may explain the presence of pheomelanic animals in this study that had an apparently similar phenotype but different genotype. When the both the Agouti signalling protein and a fully functioning receptor are present, Agouti signalling protein can act to inhibit the conversion of pheomelanin to eumelanin (Paul 1999; Hart 2001; Kerns *et al*. 2003). We suggest that a similar effect exists in alpacas, in that white-fibred, black-skinned alpacas are genetically different from white-fibred, pink-skinned alpacas.

Colour dilution genes may also account for the pigment variation displayed between animals with identical *MC1R* genotypes. Pigmentation is a multistep process with the potential for pigment to be affected at any stage (Busca and Ballotti 2000; Hoekstra 2006; Lin and Fisher 2007). Even in the presence of a fully functioning MC1R receptor, if the genes responsible for packaging and transport of melanosomes are not functional or efficient it may result in an overall dilution of the manufactured pigment merely by the inability to exit the cell and be transported into the surrounding keratinocytes (Potterf *et al*. 1998; Guibert *et al*. 2004; Le Pape *et al*. 2008).

Mutations in the promoter sequence or mutations in transcriptional elements of *MC1R* may also be responsible for the differences seen in phenotype between animals with identical *MC1R* genotypes (Furumura *et al*. 2001; Girardot *et al*. 2006). Such mutations, which affect the expression of MC1R, may explain the differing degrees of pigmentation seen in the different phenotypes. Mutations that affect the functioning of promoters and certain transcription factors have been shown to result in an increase in melanin synthesis (Murisier *et al*. 2006). GPCR promoters are characteristically GC rich and lack a TATA box (Moro *et al*. 1999). Characterisation of the *MC1R* promoter was not carried out in this study, but it is a significant area for the further investigation of fibre colour variation in alpacas.

This research has also highlighted the need to increase genetic diversity of the animals sampled. This is illustrated in a recent paper by Powell *et al*. (2008) that detailed a study of *MC1R* in alpacas in the USA. There were seven SNP common to both studies; however, four SNP that appeared in their sample population were not found in ours. It is possible that we have not identified some phenotypically relevant polymorphisms existing in the Australian alpaca population, or that reduced genetic diversity has removed some from the population. This highlights the need for further analysis of the gene before conclusions can be made about which polymorphisms are causative of colour phenotype.

This study has provided new information on the possible effects of *MC1R* alleles in alpaca fibre pigmentation. The results have highlighted a significant haplotype that appears to be a marker for the absence of black pigment. This haplotype holds significant potential for use as a marker in breeding stock selection. While this study has provided significant new information about *MC1R*, the nature of pigment gene interactions means that genetic analysis of the *Agouti* locus will be necessary before the nature of colour inheritance in this species is completely understood. Investigation and characterisation of the *MC1R* promoter may also yield useful information in analysing the differences in MC1R expression in animals with identical genotypes that display varying degrees of pigmentation.

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Appendix 1D.

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Three novel mutations in *ASIP* associated with black fibre in alpacas (*Vicugna pacos*)

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SUMMARY

The coding region of the alpaca Agouti signalling protein (*ASIP*) gene was sequenced. It was determined to be 402 nucleotides long and code for a protein that is 133 amino acids long. Eight mutations were identified in a sample of 15 alpaca, five in the coding region and three in the introns flanking the exons. *In silico* analysis showed that three of the five mutations in the coding sequence, c.325_381del57, c.292C>T and c.353G>A are probable loss-of-function mutations. The three mutations were strongly associated with black fibre colour, with 0·90 of black alpacas in the current study having two copies of one or another of the mutations. However, not all black animals displayed the putative 'aa' genotype, and almost half of the non-black animals did display that genotype. Contributing factors such as regulatory region mutations, interactions of ASIP with melanocortin-1 receptor (MC1R) and α-melanocyte stimulating hormone (α-MSH), the effect of dilution genes and subjective phenotype assignment are discussed. These mutations will allow alpaca breeders to select for or against black, but they do not explain all black phenotypes in this species.

INTRODUCTION

Colour is very important for the alpaca fibre industry because it has a substantial impact on the value of the fibre. Alpacas have more than 22 recognized colour variants and this fact, combined with the paucity of information on alpaca fibre colour inheritance patterns, and subjective phenotype recording methods, have resulted in inefficient prediction of colour outcomes in breeding programmes (Sponenberg 2001; McGregor 2006).

Pigmentation is a complex process with the potential for colour to be affected at any stage from the specification of melanocyte lineage at the neural crest to the export of melanin from the melanocyte (Hoekstra 2006; Thiruvenkadan *et al.* 2008). Although there are over 300 identified genes that have a known role in mammalian pigmentation, a few key genes have been identified as major regulators of pigment production in mammals (Rieder *et al.* 2001; Fontanesi *et al.* 2010). These are the melanocortin-1 receptor (*MC1R*), the α-melanocyte stimulating hormone (*α-MSH*) and the Agouti signalling protein (*ASIP*) genes (Sturm *et al.* 2001; Rouzaud & Hearing 2005; Hoekstra 2006). These three genes have been characterized extensively in many mammalian species including mice, humans, horses, sheep, cows, pigs and dogs, and many mutations have been reported that have associations with phenotype variation (Jackson *et al.* 1994; Rouzaud *et al.* 2000; Rieder *et al.* 2001; Sturm *et al.* 2001; Kerns *et al.* 2003).

Mammals are only able to produce two types of pigment, eumelanin and pheomelanin, which results in black to brown and red to yellow colours, respectively (Furumura *et al.* 1996; Rees 2003; Hoekstra 2006). The protein products of the *MC1R*, *α-MSH* and *ASIP* genes, and the interactions between them, control the relative amount, type and location of pigment that is produced (Furumura *et al.* 1996; Rieder *et al.* 2001; Rees 2003). Therefore, *MC1R*, *α-MSH* and *ASIP* are the genes primarily responsible for the wide array of pigment variation observed in mammals (Sturm *et al.* 2001; Hoekstra *et al.* 2006).

The product of the *MC1R* gene is the melanocortin-1 receptor protein (MC1R), which is expressed on the cell surface of melanocytes (Scott *et al.* 2002; Newton *et al.* 2005). Initiation of intracellular MC1R signalling

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is induced by the binding of its ligand, the protein product of *α-MSH*, α-MSH (Hearing 2005; Hoekstra 2006; Tully 2007). The MC1R/α-MSH complex activates the adenyl cyclase pathway that leads to increased cyclic-adenosine mono-phosphate (cAMP) levels within the cell. A high level of cAMP is a crucial factor in the initiation of downstream signalling events within the cell leading to stimulation of eumelanin production (Buscà & Ballotti 2000; Oyehaug *et al.* 2002).

The *ASIP* gene encodes for a small, secreted protein, ASIP, *c.* 133 amino acids in length which consists of a secretion signal, a lysine-rich basic region and a folded, cysteine rich C-terminus (Hustad *et al.* 1995; Willard *et al.* 1995). ASIP is an antagonist of MC1R, and acts specifically to block the activity of the MC1R agonist, α-MSH, and inhibit MC1R activation (Hustad *et al.* 1995; Willard *et al.* 1995). When the receptor is in its inactive conformation, cAMP production is reduced or inhibited, and the cell switches to pheomelanin synthesis (Furumura *et al.* 1996; Le Pape *et al.* 2008).

The *ASIP* gene consists of three coding exons, most often termed exons 2, 3 and 4. Many mammals have no reported mutations within the coding region, and in most species the coding region is highly conserved (Siracusa 1994; Chen *et al.* 1996; Graphodatskaya *et al.* 2002; McNulty *et al.* 2005; Royo *et al.* 2008; Fontanesi *et al.* 2010). However, non-agouti black in dogs and in Japanese quail is the result of mutation in exon 4 (Kerns *et al.* 2004; Hiragaki *et al.* 2008).

Recent research into alpaca fibre colour genetics has included studies on *MC1R* (Powell *et al.* 2008; Feeley & Munyard 2009). Several polymorphisms were identified in *MC1R* that may influence colour in alpacas; however, these polymorphisms were not sufficient to explain all coat colour variation observed in alpacas. There has been no reported research investigating the alpaca *ASIP* gene and its effects on fibre pigment production. Due to its close interaction with MC1R, information about ASIP is essential for gaining an understanding of the genetic mechanisms controlling colour inheritance in alpacas, an idea that is supported by the fact that alpacas exhibit phenotypes analogous to phenotypes controlled by ASIP in other species (for example black and bay in horses; black and black and tan in dogs). The objectives of the current research were to analyse the coding region of the alpaca *ASIP* gene for mutations and ascertain their potential effect on pigment production.

MATERIALS AND METHODS

Animals and DNA extraction

Blood samples were collected from 94 alpacas (Table 1). Initial sequence analysis was carried out on 15 animals (three black, two black and tan, five brown and five fawn). An additional 79 animals, comprising a wider range of colour phenotypes, were subsequently analysed, but only for exon 4 mutations. Fibre colour was determined according to the owners' assessment of the animal. Samples were collected from animals bred in the states of Western Australia, New South Wales and Victoria in Australia. Genomic DNA was extracted from 200 µl of EDTA anti-coagulated blood using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA) according to the manufacturer's instructions.

Table 1. *Colour phenotypes of alpacas used in the current study*

Fibre colour	Number of animals
Black	53
Black and tan	1
Silver-grey	1
Brown	13
Fawn	12
White	14
Total	94

Amplification and sequencing of alpaca ASIP and MC1R

Polymerase chain reaction (PCR) primers were designed to amplify the three coding exons of the alpaca *ASIP* gene (Table 2). These primers were based on the alpaca sequence assembly available on the Ensembl database (http://www.ensembl.org/index.html) and were designed to hybridize *c.* 100 bp outside of the predicted splice sites for these exons, thereby amplifying the complete coding region of alpaca ASIP, and part of the introns of the gene. All PCRs were carried out in an Eppendorf Mastercycler (Eppendorf, North Ryde, New South Wales, Australia), in 10 µl reactions containing 67 mmol/l Tris/HCl (pH 8·8), 16·6 mmol/l $[NH_4]_2SO_4$, 0·45% (v/v) Triton X-100, 0·2 mg/ml gelatin, 0·2 mmol/l dNTP (Fisher Biotec, Wembley, Western Australia, Australia), 0·2 µmol/l each of forward and reverse primer, 1 unit *Taq* DNA polymerase (Fisher Biotec), 1·5 mmol/l $MgCl_2$ and 20 ng genomic DNA. Thermal cycles were: initial denaturation at 95 °C for 3 min, followed by 30 cycles, each consisting of 94 °C for 30 s, annealing for 30 s (Table 2) and 72 °C for 45 s; with a final extension at 72 °C for 10 min. Amplified DNA was electrophoresed in 1·5% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualized by UV transillumination. The PCR products were purified using the AxyPrep PCR Cleanup Kit (Axygen). Amplification of ASIP coding exons from each animal was carried

Table 2. *Primer pairs designed for amplification of ASIP exons from genomic DNA*

Primer	Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)
Ex2F	CTCAACTGGGACACTTGTGG	416	60
Ex2R	AGCACAAAGGAGCTGTGACC		
Ex3F	TCTATTCAGCCAACCCTTCG	350	60
Ex3R	GGTCTGGTCAGAGCTCAAGG		
Ex4F	TAAGTCCGAGCAGGTAGTGG	560	65
Ex4R	AGGGAGCATGTGCGTAGC		

out in five independent 10 µl reactions, which were pooled before purification and sequencing. Sequencing reactions were carried out using ASIP primers for each exon (Table 2) with Big Dye Terminator Technology v3.1 (Applied Biosystems, Mulgrave, Victoria, Australia) and analysed on a 3730 DNA analyser (Applied Biosystems). Genotypes at *MC1R* were obtained (following the procedures outlined in Feeley & Munyard 2009) for non-black alpacas in this study who exhibited putative non-functional ASIP mutations.

Sequence assembly and analysis

Splice sites were determined using the program SpliceView (http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html; verified 19 Nov 2010) coupled with the known bovine and human ASIP exons. Complete ASIP coding region sequences for each animal were compiled using Geneious software (Biomatters, Auckland, New Zealand), and were compared with genes and proteins from other species by GenBank NCBI BLASTn and BLASTx protocols (http://blast.ncbi.nlm.nih.gov/Blast.cgi; verified 19 Nov 2010).

Genotyping ASIP mutations

Once mutations were identified in alpaca ASIP (Table 3) an additional sample group was genotyped for the four exon 4 polymorphisms (Table 4). The PCRs were carried out as above on an additional 79 animals (Table 1). Amplified DNA was electrophoresed in 1·5% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualized by UV transillumination. PCR products were then sequenced using Big Dye v3.1 on a 3730 DNA analyser (Applied Biosystems) at Macrogen Inc., South Korea, using primers Ex4F and Ex4R. The deletion was analysed by electrophoresis in 2% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualized by UV transillumination. Fisher's exact test was used to test for non-random association between mutations and colour.

Table 3. *Polymorphisms identified in the alpaca* ASIP *gene*

Polymorphism	Location	Amino acid effect
c.102G>A	Exon 2	Synonymous
c.291C>A	Exon 4	Synonymous
Exon 3 +34C>T	Intron 2	N/A
Exon 3 +56A>C	Intron 2	N/A
Exon 4 −41C>A	Intron 2	N/A
c.292C>T	Exon 4	R98C
c.353G>A	Exon 4	R118H
c.325_381del57	Exon 4	C109-R127del
Exon 4 +10C>T	3′ UTR	N/A
Exon 4 +38A>G	3′ UTR	N/A

Predictive modelling of ASIP

A multiple alignment of ASIP sequences from 32 different mammalian species was performed using Jalview 2.0 (Waterhouse *et al.* 2009) with the Muscle algorithm (Edgar 2004) using default values. The web server versions of the scale-invariant feature transform (SIFT; Kumar *et al.* 2009), iMutant 2.0 (Capriotti *et al.* 2005), iPTree-STAB (Huang *et al.* 2007) and PolyPhen (Ramensky *et al.* 2002) algorithms were used to predict the potential effect of amino acid substitutions on ASIP structure and function. Default values were used for all programs. SIFT is based on sequence conservation and homology, using position-specific scoring matrices. It predicts the effects of amino acid substitution on both structure and function. iMutant 2.0 and iPTree-STAB use a thermodynamic method and base their predictions on protein stability using the calculated free energy change ($\Delta\Delta G$) of mutations within the protein sequence. PolyPhen bases its predictions on empirical rules applied to the protein's sequence, phylogenetic and structural information. All methods, except iPTree-STAB, provide an estimate of confidence in their predictions. The signal peptide was detected using the web servers of SignalP (Bendtsen *et al.* 2004), CoSiDe (http://sigpep.services.came.sbg.

Table 4. *ASIP genotypes of the three significant exon 4 polymorphisms examined in the current study*

Putative ASIP genotype	Exon 4 genotype c.292C>T R98C	c.353G>A R118H	c.325_381del57 C109_R127del	Colour	Number of animals
a^1a^1	CC	–	Yes	Black	21
				Brown	3
				Fawn	1
a^2a^2	TT	GG	No	Black	4
a^3a^3	CC	AA	No	Black	5
				Brown	2
				White	1
AA	CC	GG	No	Fawn	1
				White	5
a^2a^3	CT	GA	No	Black	3
				Brown	1
				Fawn	1
				White	2
Aa^3	CC	GA	No	Fawn	5
				White	3
Aa^2	CT	GG	No	Black	1
				Brown	1
				Fawn	1
				White	1
Aa^1	CC	G	Het	Black	4
				Silver-grey	1
				Brown	2
				Fawn	2
				White	1
a^1a^3	CC	A	Het	Black	2
				Brown	2
a^1a^2	CT	G	Het	Black	13
				Black and tan	1
				Brown	2
				Fawn	1
				White	1

ac.at/; verified 19 Nov 2010) and Phobius (Kall *et al.* 2007).

RESULTS

The alpaca ASIP *gene*

The complete coding sequence of the alpaca *ASIP* gene was generated (GenBank accession no. HM768322). Homology with the *ASIP* gene of other species was confirmed, with the highest sequence homology being to cow, goat and sheep (89%) and pig (88%). Exon 2 is 160 bp, exon 3 is 65 bp and exon 4 is 177 nucleotides in length. The entire coding region is 402 bp long, 6 bp longer than the dog and mouse, 3 bp longer than the human and 6 bp shorter than the cat ASIP coding region. A consensus splice acceptor and splice donor site flanks each coding exon. Exons 2, 3 and 4 contain 54, 40 and 68% GC (respectively) and the whole coding region is 58% GC.

The predicted alpaca ASIP protein

Alpaca *ASIP* translates into a 133 amino acid predicted protein (Fig. 1) that is 0·83 identical to sheep and cow ASIP and 0·81 identical to the horse and rabbit proteins (see end of this paper for link to supplementary information). The consensus prediction for the signal peptide in ASIP was from residues 1 to 22 by two out of three signal peptide prediction methods. The predicted peptide cleavage site was between residues 22(S) and 23(H). In contrast, Phobius predicted a signal peptide cleavage site between residues 24 and 25.

A pairwise alignment of the alpaca ASIP sequence with the sequence of an engineered ASIP structure (Protein Data Base ID: 2KZA) showed that they were 0·74 identical in the 53 residue, cysteine rich, C-terminal sequence. All cysteines in the pairwise alignment were exactly aligned. Consequently, it can be inferred by sequence similarity that the disulphide

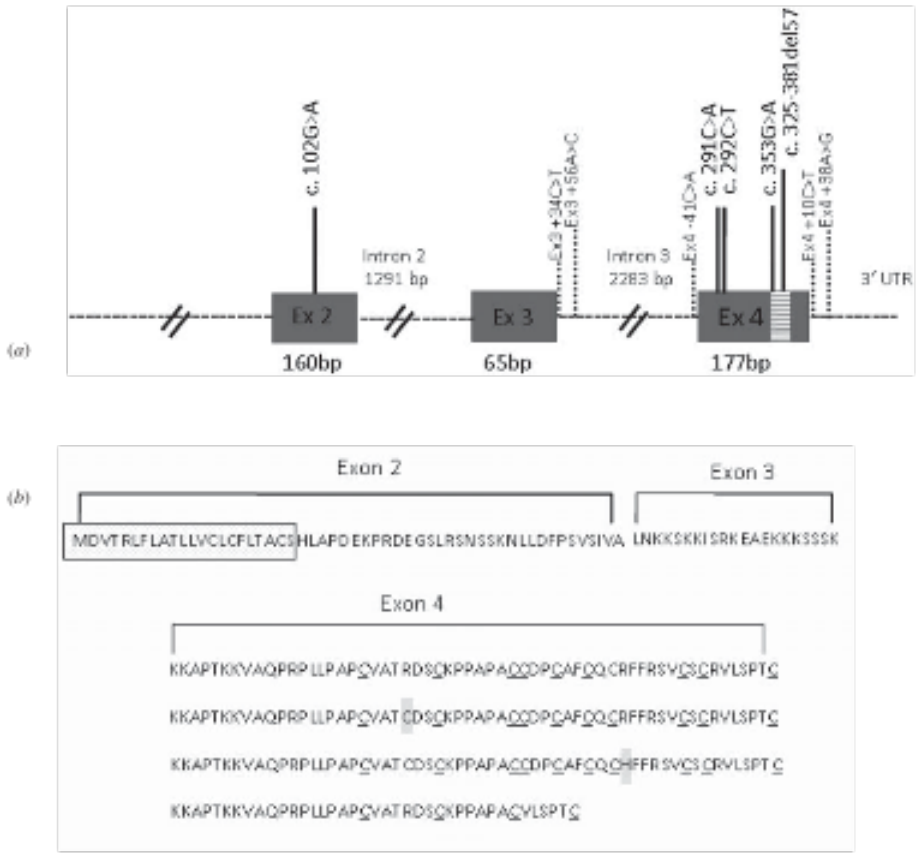

Fig. 1. The alpaca *ASIP* gene. (*a*) Coding exons 2, 3 and 4 are shown as solid boxes. Untranslated regions are shown as dashed lines (not to scale). Polymorphisms are shown in the regions where they occur. Indicated intron size was determined from the Ensembl genome assembly. (*b*) The predicted alpaca wild-type ASIP protein. The conserved cysteine residues in the C-terminus are underlined, the secretion signal is shown as a dotted box. Predicted exon 4 protein sequences for the R98C, R118H (grey boxes) and C109_R127del mutations are shown below the wild-type protein.

bonds in alpaca ASIP are likely to occur at cysteine residue locations [94–109], [101–115], [108–126], [112–133] and [117–124]. The disulphide bond prediction method DBCP (Lin & Tseng 2010) also predicted disulphide bonds at exactly the same locations in alpaca ASIP.

Mutations in alpaca ASIP and MC1R

Sequencing of the *ASIP* coding region in 15 alpacas revealed five polymorphisms (Table 3). Two non-synonymous single nucleotide polymorphisms (SNPs) were identified in exon 4: c.C292T, predicted to cause an arginine-to-cysteine substitution at codon 98 (R98C; GenBank accession no. HM768323), and c.G353A, predicted to cause an arginine-to-histidine substitution at codon 118 (R118H; GenBank accession no. HQ008273). A 57 bp deletion was also discovered in exon 4. This deletion, occurring at nucleotides 325–381 (c.325_381del57; GenBank accession no. HM768324), is predicted to result in 19 of the last 25 amino acids being absent from the mature protein (p.C109_R127del). In addition, two synonymous mutations; c.G102A in exon 2 (p.G34) and c.C291A in exon 4 (p.T291) were identified. Four haplotypes involving these mutations were observed in this group of alpacas, and these haplotypes existed in ten different combinations (Table 4). While no match occurred between any fibre colour and any single *ASIP* genotype in the 94 animals studied (Table 4) there was strong association between the homozygous state of each mutation and black colour,

as well as between the combined alleles and black colour (Fisher's exact test for c.325_*381del57* $P<0{\cdot}001$; for c.292C>T $P=0{\cdot}039$; for c.353G>A $P=0{\cdot}024$; and for all combined $P<0{\cdot}001$). Forty eight of the 53 black animals were either homozygous for C109_R127del (allele a^1), the T allele of R98C (a^2), the A allele of R118H (a^3) or were heterozygous for a combination of two of these mutations. However, 18 of the 41 non-black animals also exhibited some of these same genotype combinations. These animals exhibited a range of MC1R genotypes (Table 5). An additional five SNPs were identified in the non-coding regions flanking the exons (Table 3).

Predicted functional effect of ASIP mutations

The arginine residues at positions 98 and 118 in the alpaca ASIP sequence are highly conserved in all ASIP sequences aligned from 32 different mammalian species (see end of the paper for link to supplementary material). All amino acid substitution prediction methods used predicted that the R98C and R118H amino acid substitutions are potentially damaging to ASIP structure or function (Table 6). Only the SIFT method labelled its prediction as 'low confidence'. Therefore, it is proposed that the mutations R98C, R118H and C109-R127del are all loss of function non-agouti equivalent 'a' mutations for alpaca ASIP.

DISCUSSION

Three novel mutations in exon 4 of the alpaca *ASIP* gene have been identified that are each predicted to cause a loss of function in the protein. Exon 4 codes for the last 40 amino acid residues of ASIP, which constitute the C-terminal domain and the majority of the residues are responsible for protein activity and receptor binding (Dinulescu & Cone 2000; Miltenberger *et al.* 2002). It has been reported that the ten cysteine residues within the C-terminus are involved in a network of five disulphide bonds that acts to stabilize the protein (McNulty *et al.* 2005; Yu & Millhauser 2007) and form a unique fold motif known as an inhibitor cysteine knot (ICK; McNulty *et al.* 2005; Yu & Millhauser 2007). The spacing of the ten cysteine residues in ASIP is strictly conserved throughout all mammals that have been investigated (Miltenberger *et al.* 2002; McNulty *et al.* 2005; Yu & Millhauser 2007). The particular fold structure of the ICK allows for presentation of three important conserved residues, $Arg_{116}Phe_{117}Phe_{118}$, in order to facilitate MC1R interaction and binding (Miltenberger *et al.* 2002). This structure can therefore be assumed to be essential for correct ASIP functioning. Animals carrying C109_Rdel19 (aka a^1) are missing six of these ten conserved cysteine residues. This would almost certainly prevent tertiary structures, such as the ICK, from being formed correctly. With this type of disruption to the protein it is almost certain that function is eliminated.

About half of all known disease-causing mutations result from amino acid substitutions, and automated prediction methods can be used to identify potentially damaging substitutions (Ng & Henikoff 2006). The current study used four automated methods to derive a consensus view on the potential effect of the two observed amino acid substitutions on ASIP structure

Table 5. *MC1R Genotypes of non-black animals with aa genotypes*

Putative ASIP genotype	Colour	Number of animals	MC1R genotype
a^1a^1	Brown	3	EE, Ee, ee
	Fawn	1	Ee
a^2a^2	Brown	2	Ee ee
	White	1	Ee
a^2a^3	Brown	1	Ee
	Fawn	1	Ee
	White	2	Ee Ee
a^1a^3	Brown	2	Ee ee
a^1a^2	Black and tan	1	Ee
	Brown	2	Ee ee
	Fawn	1	ee
	White	1	EE

Table 6. *Potential effect of amino acid substitution on ASIP structure or function*

Amino acid substitution prediction method	Amino acid substitution	
	R98C	R118H
SIFT	Likely to affect function (*low confidence*)	Likely to affect function (*low confidence*)
iMutant 2.0	Decrease in stability of structure (*high confidence*)	Decrease in stability of structure (*high confidence*)
iPTree-STAB	Destabilizing* to structure	Destabilizing* to structure
PolyPhen	Probably damaging* to structure and function (*high confidence*)	Possibly damaging* to structure and function (*medium confidence*)

* The terms 'destabilizing', 'probably damaging' and 'possibly damaging' are used by the respective methods to describe the effect of the amino acid substitution.

and function. It is preferable to use more than one prediction method, based on different assumptions and algorithms, because this gives increased support for any inference that may be made on the functional or structural significance of an amino acid substitution. In all cases the automated methods predicted a potentially damaging or destabilizing effect on ASIP structure or function for both SNPs (Table 6). Only the SIFT method labelled its prediction as 'low confidence'. The SIFT method depends on sequence diversity to generate confidence values and there was insufficient sequence diversity in the sequence alignment generated by SIFT (which uses PsiBlast to find and align, functionally related sequences). This is a common issue with prediction algorithms that depend on sequence conservation and is understandable given the highly conserved nature of the ASIP sequence.

There are a number of ways in which these SNPs might cause a loss of function in ASIP. The R98C polymorphism results in the substitution of a cysteine for a histidine in the highly conserved C-terminus of the protein. Previous studies have reported that the loss of even one of these highly conserved cysteine residues is sufficient to abolish the activity of the protein (Perry *et al.* 1995; Miltenberger *et al.* 2002). The addition of another cysteine residue in this region is predicted to disrupt the formation of this essential tertiary structure and be responsible for a loss of function of the protein. Protein alignment results suggest that the R98C polymorphism in alpacas is the same as the R96C polymorphism present in dogs (Kerns *et al.* 2004), due to the upstream difference of two amino acids between the two proteins. The R96C mutation in dogs has been shown to be a loss of function mutation resulting in non-agouti black (Kerns *et al.* 2004). The similarity between these polymorphisms further supports the current *in silico* predictions that R98C has a similar affect in alpacas.

The residues $Arg_{116}Phe_{117}Phe_{118}$ have an essential role in protein interactions of the C-terminal domain (Miltenberger *et al.* 2002; McNulty *et al.* 2005). When correctly folded, these are the binding determinants of the protein and they facilitate direct interaction with Melanocortin receptors (Miltenberger *et al.* 2002; McNulty *et al.* 2005). This suggests that the amino acid sequence of this domain and the correct structural folds are important determinants for protein function. The R118H substitution is usually considered a conservative substitution according to the Blosum62 evolutionary matrix (Henikoff & Henikoff 1992) and this is understandable given the similar physicochemical properties of arginine and histidine. However, any change in a highly conserved region has the potential to be detrimental to the protein's structure or function. The conserved $Arg_{116}Phe_{117}Phe_{118}$ amino acids are homologous to residues $Arg_{118}Phe_{119}Phe_{120}$ in alpaca ASIP. Therefore, the R118H mutation results in a His-Phe-Phe combination present in the active loop (instead of the conserved Arg-Phe-Phe combination) which may prevent ASIP interaction with MC1R, resulting in a loss-of-function variant. However, functional studies, such as testing the effect of these proteins on receptor signalling *in vitro* or RNAi in mouse models, are needed to confirm that these mutations are affecting ASIP function. It is interesting to note that the three exon 4 mutations probably occurred in historically independent populations. The R118H mutation cannot exist in combination with C109_T127del19, and the T allele of R98C was never found in combination with the deletion, nor with the A allele of R118H. Only four haplotypes were present in the sample of alpacas used in the current study: C,G,No, C,Yes, T,G,No and C,A,No. Black alpacas are considered to be sacred in South America, and are still sacrificed to the Gods (Bolin 1998; J. C. Wheeler, personal communication). Therefore, historically, any new mutations causing black animals to occur would have been preserved by selective breeding.

It would be expected that animals that are homozygous for any of the non-functional *ASIP* alleles would have a black phenotype. This is largely consistent with the current data (Table 4): 48 out of 53 black animals were homozygous for a^1, a^2 or a^3, or were heterozygous for two of these alleles. The five animals that were black in phenotype but did not carry two putative black alleles could have as yet undiscovered mutations in ASIP exons 2 or 3, regulatory mutations leading to decreased ASIP expression, or dominant mutations at another gene (e.g. MC1R or β-defensin).

Almost half of the non-black animals were homozygous for the putative non-functional mutations. There are four reasons why non-black animals might carry a black genotype. The first is that a non-functional *MC1R* genotype is also present (i.e. ee), thus the specific *ASIP* allele is irrelevant. MC1R and ASIP have an epistatic relationship where a fully functioning MC1R receptor is necessary for the *ASIP* alleles to be expressed (Furumura *et al.* 1996; Hoekstra 2006). If MC1R is non-functional then it cannot be activated or inactivated by either of the two alternate ligands. It will therefore only express pheomelanin, and the *ASIP* alleles will be masked. Just over one-third of the non-black 'aa' animals are homozygous for the putative non-functional *MC1R* allele 'e' (Table 5; Feeley & Munyard 2009); however, others were not. Secondly, if α-MSH is also non-functional, then MC1R will receive no signal from either its agonist or antagonist, and will revert to pheomelanin production.

A third possible explanation is that dilution genes are having an effect on the underlying colour. The dilution genes *MATP* and *TYR* have been investigated in alpacas, but no mutations were found that

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